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VOL. 46 AUGUST, 1955 NO. 2

CLINICAL AND EXPERIMENTAL

STUDIES ON AGAMMAGLOBULINEMIA

II. FAILURE OF PLASMA CELL FORMATION IN THE BONE MARROW AND LYMPH NODES OF PATIENTS WITH AGAMMAGLOBULINEMIA

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MINNEAPOLIS, MINN.

MUCH has been written in recent years concerning the cellular basis of antibody and gamma globulin production. Impressive evidence indicates that synthesis of both antibodies and gamma globulins may be extrahepatic.[1] Conclusive proof has been presented demonstrating that lymph nodes form antibodies in response to antigenic stimulation[2, 3] and data have accumulated indicating that both spleen and bone marrow also participate in this physiologic activity.[4, 5] Currently, argument focuses on the specific cell type responsible for the execution of antibody synthesis.[1] Evidence at one time or another has been presented implicating cells of the reticuloendothelial system,[6, 7] lymphocytes,[8, 9] and, more recently, plasma cells[4, 5, 10, 11] in this activity. Data presented by Reiss and associates,[12] Moeschlin and co-workers,[13] and Coons and associates[14] seem to be sufficiently direct to establish that plasma cells are one of the cellular sources of antibody production in experimental animals. In spite of these data, there are still those who express doubt as to this relationship or who advocate other cells as the source of humoral antibody and gamma globulin.

Clinical, pathologic, and hematological studies in human beings[15-17] also provided abundant evidence which links plasma cell formation to the elaboration of gamma globulin and the synthesis of antibody. Almost all of these data have been of a correlative nature and support a positive relationship.[18] For example, in numerous different diseases with apparently various pathogenic

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mechanisms, increased numbers of plasma cells have been demonstrated to exist in the tissues of the body in the presence of increased concentrations of gamma globulin or antibody in the circulation. Even a more critical association was demonstrated in rheumatic fever[17] where it was found that the absolute number of plasma cells in the bone marrow was precisely correlated with the slope of the curve of antibody and gamma globulin accumulation in the serum. Of value in establishing or rejecting in human beings the directness of the relationship between antibody and gamma globulin production on the one hand and plasma cell proliferation on the other would be a study of the hematopoietic tissues in a disease characterized by a decrease in gamma globulin or antibody formation. With the discovery of agammaglobulinemia, a clinical disease featured by isolated failure of gamma globulin and antibody synthesis, this opportunity exists.

Recently Bruton[19] has described a group of patients whose principal abnormality is a deficiency of both antibody and gamma globulin production. This disease has been called agammaglobulinemia and has been well defined by Janeway and co-workers.[20] The patients with agammaglobulinemia are characterized clinically by their extreme susceptibility to bacterial infections. The diagnosis can readily be made by electrophoretic analysis of their plasma or serum. In addition to lacking gamma globulin in their blood and tissues, these patients are unable to produce antibodies, and it has been reasoned that they are suffering from a profound disturbance of the mechanism responsible for synthesis or release of antibodies and gamma globulin.[21]

Clinically, agammaglobulinemia occurs in two forms, a congenital disease sometimes familial in nature which probably represents an inborn error of metabolism transmitted as a sex-linked recessive trait and an acquired form which occurs sporadically at any age in either sex.

Study of patients suffering from agammaglobulinemia has provided a new and valuable approach to certain fundamental immunologic problems. Along with other investigations in these patients, it seemed reasonable to attempt to gain evidence concerning the cellular basis of antibody and gamma globulin formation by studying the hematopoietic tissues of patients who can form neither antibodies nor gamma globulin. We have found that both children and adults suffering from agammaglobulinemia have a striking deficiency in the numbers of plasma cells in their hematopoietic tissues. Furthermore, unlike normal persons, patients with this form of immunologic and metabolic derangement do not develop plasmacytosis in response to even the most intensive antigenic stimulation. It is these observations which occasion the present report.

MATERIALS AND METHODS

The subjects of the present investigation include eleven essentially normal children ranging in age from 6 to 12 years and 4 patients with agammaglobulinemia. The agammaglobulinemic patients are two children with congenital disease and two adults with the acquired illness. The children were both 7-year-old boys. Each had been troubled from the age of 6 months with recurrent severe bacterial infection; for example, bacterial diarrhea, recurrent pneumonia, recurrent bacterial meningitis, recurrent urinary tract infection, and recurrent severe infections of the upper respiratory tract.

Extensive immunologic studies[24] revealed that both children were suffering from a virtually complete paralysis of the immunologic mechanism. Even the so-called natural isohemagglutinins were lacking from the circulation and could not be demonstrated following challenging injections of large volumes of washed red blood cells of heterologous blood groups.

In the other two patients with agammaglobulinemia, convincing clinical evidence established the acquired nature of their illness. One patient, L. L.,* was a 30-year-old woman who was well until she was 22 years old. During the eight years prior to study, she had been inordinately susceptible to bacterial infections, having had 34 attacks of pneumonia, often lobar in character, which frequently proved to be due to pneumococcal infection, 3 episodes of pneumococcal meningitis, numerous attacks of sinusitis, repeated episodes of respiratory disease, urinary tract infection, and infection of the gastrointestinal tract. The other patient was a 58-year-old man who had been ill only four years prior to study. He, too, had been troubled by extreme susceptibility to bacterial infection. Having been essentially without serious illness until 54 years of age, he had had 17 attacks of pneumonia, proved on several occasions to be due to pneumococci, and many other bacterial infections during the four years prior to admission to the hospital.

The children referred to as normal in this study were children known to be free of recognizable metabolic or infectious disease who were seen at the University of Minnesota Hospitals for surgical correction of minor congenital anomalies, evaluation of behavior disorders, or investigation of mental retardation. In no instance was a patient included in this group whose basic disturbance could conceivably influence the hematopoietic tissues or the production of antibody.

Bone marrow samples from the children were taken from the iliac crest or the body of the sternum, the site of aspiration being a function only of convenience. This is possible since significant differences in the marrow samples obtained from these sites in children cannot be demonstrated.[26] The aspiration biopsies were taken and the smears prepared and analyzed according to methods previously described.[17]

Lymph nodes were removed surgically from the inguinal region, local anesthesia being used to permit their excision: Imprints, so-called touch preparations, were prepared by dividing the fresh node longitudinally with a previously unused razor blade and touching the surface of the node with a clean glass slide. Fixation of the imprints was achieved by drying the slide rapidly in the air. Romanovsky stains, particularly the Wright-Giemsa combination, were used in preparation of the slides for microscopic examination. After imprinting, the nodes were placed in 10 per cent formol-saline and prepared for histologic examination in the usual fashion.

Lymph nodes were obtained from the agammaglobulinemic patients prior to antigenic stimulation, four days following primary exposure to typhoid-paratyphoid antigens and four days following a later secondary exposure to the same antigens. Similarly, in the normal children lymph nodes were obtained prior to antigenic stimulation in 4 instances, following primary antigenic stimulation in 5 instances, and during the secondary response in 4 instances.

In the experiments in which the lymph nodes were removed following antigenic stimulation, typhoid-paratyphoid antigens were injected both intradermally and subcutaneously into the anterior medial aspect of the thigh in a total dose of 1.0 ml. The vaccine used was Lederle typhoid-paratyphoid vaccine for immunization containing 1,000 million B typhosa, 250 million paratyphi A, and 250 million paratyphi B organisms per milliliter.

Marrow biopsies were taken prior to the initial injection of antigen and again following a series of subcutaneous and intravenous injections of T.A.B. organisms. The total number of organisms injected prior to the second marrow biopsy was 1,000 million typhoid organisms, 500 million paratyphoid organisms intradermally, 1,000 million typhoid, 500 million paratyphoid organisms subcutaneously, and 200 million T.A.B. organisms intravenously over a three-week period.

In one of the agammaglobulinemic patients, bone marrow biopsies were obtained serially following three weeks of typhoid-paratyphoid stimulation, ten days following administration

*A case report describing this patient has been published.[25]

of "mismatched" red blood cells, four weeks after application of a homologous skin graft from an unrelated person, and one week following the second injection of a group of virus antigens including the following: 1 ml. Lederle mumps vaccine, 1 ml. Salk poliomyelitis vaccine, 1 ml. influenza vaccine,* 1 ml. each of Rocky Mountain spotted fever vaccine,† Q fever vaccine,† and typhus vaccine.†

Bone marrow plasma cell content was evaluated according to techniques previously described,[17] stable figures for the numbers of plasma cells being regularly obtained when as many as 5,000 nucleated marrow cells were counted.

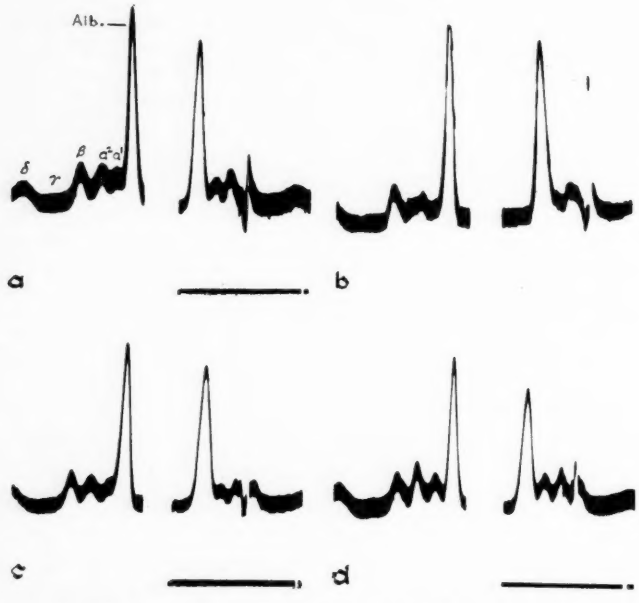

Fig. 1.—Electrophoretic patterns on each of four patients with agammaglobulinemia. Note the complete absence of gamma globulin as determined electrophoretically and the normal concentrations of the other protein components of the serum in each instance.

OBSERVATIONS

On Fig. 1 are shown the electrophoretic patterns of the two children (Fig. 1, *a* and *b*) and two adults with agammaglobulinemia (Fig. 1, *c* and *d*). The diagnostic feature, namely, the complete absence of gamma globulin, is apparent in each of these patterns.

In Table I the plasma cell content of the marrows of a group of 12 normal children and 15 normal adults are compared to the plasma cell content of the marrows of the 4 patients with agammaglobulinemia and to the plasma cell content of the bone marrow of each of 4 children chosen to be subjected to intensive antigenic stimulation. It may be seen from the data presented in the table that the bone marrow of patients with agammaglobulinemia is grossly deficient in plasma cells. Indeed, the virtual absence of plasma cells in the marrows

*Lederle vaccine prepared from the extraembryonic fluid of the infected chick embryo, Lot No. 2035-125A.

†Kindly supplied by Dr. Karl Eklund, Rocky Mountain Spotted Fever Research Laboratories, Hamilton, Montana.

of three of the patients and the extreme reduction of their numbers in a fourth permit a qualitative separation of the marrows of these patients from those of normal persons or persons suffering from other diseases.

TABLE I. NUMBER OF BONE MARROW PLASMA CELLS IN NORMAL AND AGAMMAGLOBULINEMIC PATIENTS

	PATIENT	AGE (YR.)	SEX	PLASMA CELLS PER 5,000 NUCLEATED MARROW CELLS		
				MEAN	RANGE	NUMBER 5,000 CELL COUNTS
Normal persons	12 Normal children	2½-15	–	19.8	11-32	12
	15 Normal adults	18-41	–	31.0	8-61	15
	R. L., plastic repair of burn scar	10	M	25.1	19-32	4
	W. O., cleft lip and palate	7	M	11.9	9-17	4
	R. A., mental retardation	7	F	18.0	12-29	4
	K. O., postencephalitic brain damage	7	F	23.5	17-29	4
Patients with agammaglobulinemia	E. S.	7	M	0.25	0-1	4
	W. A.	7	M	0.00	–	4
	L. L.	30	F	0.00	–	4
	F. H.	58	M	1.0	0-3	4

TABLE II. BONE MARROW CELLS FOLLOWING ANTIGENIC STIMULATION IN NORMAL CHILDREN AND IN PATIENTS WITH AGAMMAGLOBULINEMIA

PATIENT	PLASMACYTES PER 20,000 NUCLEATED MARROW CELLS		TYPHOID (H) AGGLUTININ TITER	
	BEFORE ANTIGENIC STIMULATION	AFTER ANTIGENIC STIMULATION	BEFORE STIMULATION	AFTER STIMULATION
Normal children				
R. L.	104	530	No titer	1-20,480
W. O.	47	269	No titer	1-10,240
R. A.	72	312	No titer	1-10,240
K. O.	94	515	No titer	1-20,480
Patients with agammaglobulinemia				
E. S.	1	0	No titer	No titer
W. A.	0	0	No titer	No titer
L. L.	0	2	No titer	No titer
F. H.	5	7	No titer	No titer

In Table II the response of the plasma cell content of the marrow to intensive stimulation with typhoid-paratyphoid antigens is compared in four normal children and in four patients with agammaglobulinemia. In each of the normal children, a significant plasmacytosis associated with a marked increase in agglutinin titer occurred as a consequence of the antigenic stimulation. In striking contrast, concomitant failure of antibody production and bone marrow plasma cell development characterized the patients with agammaglobulinemia. The morphologic abnormality in the bone marrow is illustrated in Figs. 2 and 3 in which photomicrographs compare typical areas from the marrow of a patient with agammaglobulinemia to that of a normal child after comparable antigenic

exposure. Just as has been repeatedly demonstrated in experimental animals,[4, 5, 11] numerous plasma cells occurred in the marrows of normal children during a period of active antibody production. In contrast, these cells were almost completely absent from the marrows of patients suffering from agammaglobulinemia regardless of the intensity of the antigenic stimulation.

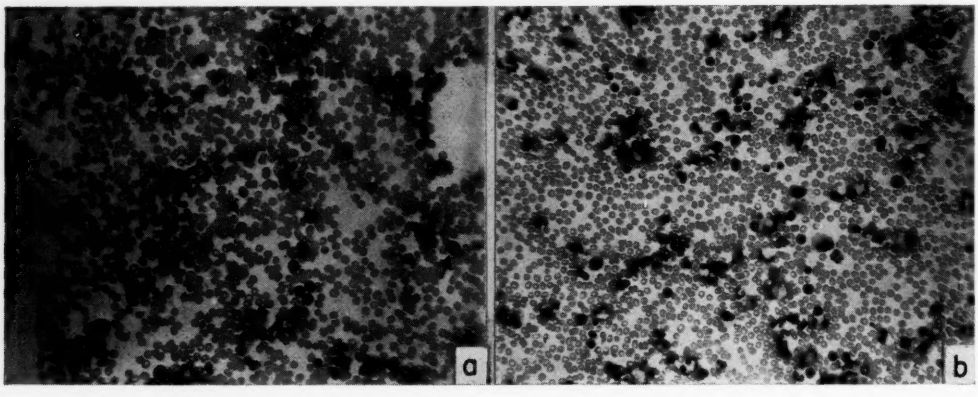

Fig. 2.—Comparison of the bone marrows from (*a*) a normal child and (*b*) a child suffering from agammaglobulinemia following intensive antigenic stimulation. Note the abundance of plasma cells in the marrow of the normal child and their absence from the marrow of the patient with agammaglobulinemia. (×100; reduced ⅓.)

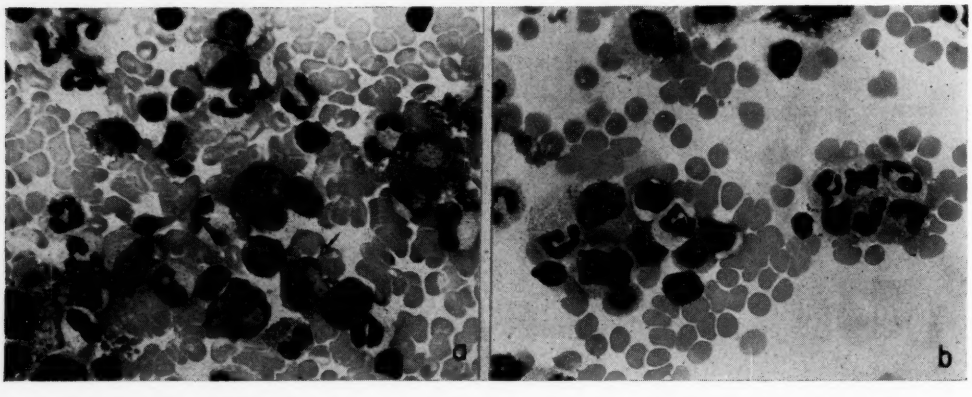

Fig. 3.—High-power view of plasma cells and developing plasma cells from bone marrow of normal child undergoing intensive antigenic stimulation with typhoid-paratyphoid organisms (*a*). High-power view of comparable area from the marrow smear of the agammaglobulinemic subject (*b*) illustrating absence of plasma cells. (×800; reduced ⅓.)

In Table III are presented data obtained from serial marrow biopsies demonstrating that the antigenic stimulus provided by the intravenous injection of mismatched red blood cells, the grafting of homologous skin, injection of diphtheria toxoid, pertussis organisms, and tetanus toxoid, simultaneous immunization with antigens derived from polio virus, *Rickettsia rickettsii, R. prowazekii,* Q fever organisms, mumps virus, and influenza virus produced neither a plasma cell response in the marrow nor evidence of antibody formation.

Comparison of the Morphology of Lymph Nodes in Patients With Agammaglobulinemia to Those of Normal Children.—In the gross, the lymph nodes removed from 3 of the 4 patients with agammaglobulinemia were relatively small and, on section in the fresh state, appeared to be more fibrous than normal. The node removed from the agammaglobulinemic adult woman on the other hand was large, fleshly, and firm. On microscopic study, the abnormalities in lymph node structure of all the patients with agammaglobulinemia included relative thinness of the cortex, relative deficiency in primary lymph follicles, and relative abundance of the fibrous tissue and reticulum. Cytologic investigation revealed that, except for plasma cells, all cell types seen in the nodes of normal children were to be found in the nodes of patients with agammaglobulinemia. Extensive search through the imprint and section material failed to reveal plasma cells in the nodes of these patients. Plasma cells present in small numbers were regularly found in the nodes of normal children. In Fig. 4 an inguinal lymph node from a patient with agammaglobulinemia is compared to that of a normal child.

TABLE III. PLASMA CELLS IN BONE MARROW OF PATIENT WITH AGAMMAGLOBULINEMIA FOLLOWING VARIOUS ANTIGENIC STIMULI

ANTIGENIC CHALLENGE	RESPONSE TO CHALLENGE	PLASMA CELLS PER 5,000 NUCLEATED MARROW CELLS
Following 3 weeks of typhoid-paratyphoid stimulation	No antibody titer developed	0
Ten days following administration of 15 c.c. of mismatched red blood cells	No isoagglutinins produced	1
Four weeks after application of homologous skin graft	Successful homotransplantation of skin	1
One week after second injection of series of virus antigen	No evidence of antibody formation against any of antigens	0
Diphtheria, pertussis, tetanus vaccine	No antibody production against diphtheria and pertussis antigens	0

Response of Lymph Nodes to Antigenic Stimulation.—As shown in Table IV, following antigenic stimulation, hypertrophy of the lymph nodes occurred in both normal children and patients with agammaglobulinemia. In the normal children changes recorded in the nodes included proliferation of lymphocytes through apparent increase in activity of the germinal centers, cytoplasmic budding of lymphocytes, proliferation of reticulum cells, and significant plasma cell proliferation. The formation of secondary follicles was a prominent feature. The increased number of plasma cells had accumulated in clusters throughout the node, but particularly in the medullary cords. Morphologic evidence indicated that these cells were developing largely by heteroplastic metamorphosis primarily from the reticulum and from hematopoietic reticular cells (mesenchymatous elements) also from the very basophilic reticular lymphocytes of Sundberg.[27]

Following antigenic stimulation, the nodes of the patients with agammaglobulinemia showed gross hypertrophy, some lymphocytic proliferation, a considerable degree of cytoplasmic budding of the lymphocytes, and proliferation of the cells of the reticulum, but no evidence of plasma cell formation. In

Fig. 4.—Comparison of an inguinal lymph node of a normal child (*a*) with that of a patient with agammaglobulinemia (*b*), prior to antigenic stimulation. Note the relative thinness of the cortex and abundance of fibrous tissue in the nodes from the patient with agammaglobulinemia. The cortex of the normal lymph node is considerably thicker and contains several primary lymphoid follicles.

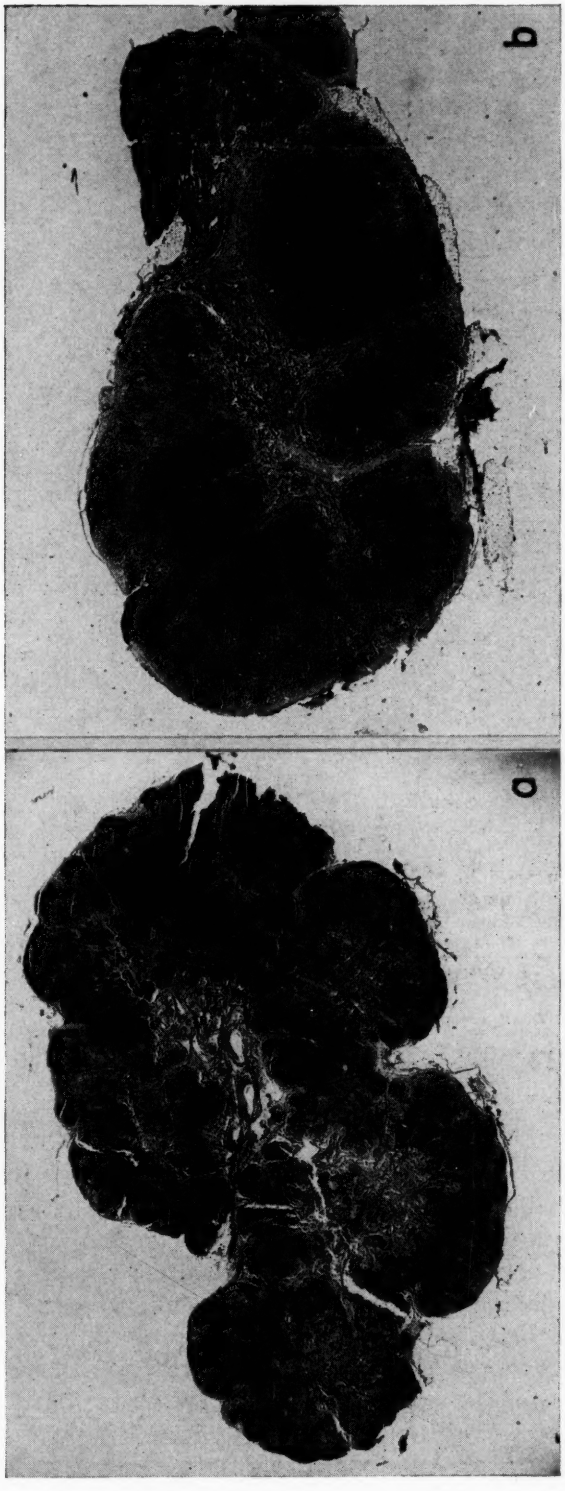

Fig. 5.—Comparison of an inguinal lymph node from a patient with agammaglobulinemia (*b*) and a normal child (*a*) taken from the right groin four days after the initial injection of 1.0 ml. T.A.B. bacterial antigen into the right thigh. The relative thickness of the cortex and the abundant germinal activity of the normal nodes stand in contrast to the relatively thin cortex, the minimal germinal activity, and the abundant fibrous tissue from the patient with agammaglobulinemia.

addition, notable failure of secondary follicle formation characterized the nodes of the agammaglobulinemic patients. Study of both imprint and section material failed to reveal the presence of plasma cells in significant number. Similar, but even more striking, differences were noted following secondary exposure to antigen. These findings are summarized in Table IV where the lymph nodes of normal children are compared to those of patients with agammaglobulinemia before antigenic stimulation, following initial exposure to antigen, and during the secondary response to the same antigen.

TABLE IV. MORPHOLOGIC RESPONSE OF LYMPH NODES FROM NORMAL AND AGAMMAGLOBULINEMIC PERSONS TO ANTIGENIC STIMULATION

GROUP		PATIENT	LYMPHNODE SIZE	PROLIFERATION OF LYMPHOCYTES	CYTOPLASMIC BUDDING OF LYMPHOCYTES	PROLIFERATION OF RETICULAR CELLS	PLASMA CELL ACCUMULATION
Before injection of antigen	Normal children	1	+	+	+	+	±
		2	+	+	+	−	±
		3	++	++	−	+	+
		4	+	++	+	+	±
	Agammaglobulinemic patients	E. S.	+	±	+	+	−
		W. A.	+	+	+	+	−
		L. L.	+++	±	++	++++	−
		F. H.	++	±	+	++	−
Primary response 4 days after first injection of antigen	Normal children	4	++++	++	+++	+++	++
		6	+++	+++	+++	+++	++
		7	++	++	++++	++	+
		8	++	++	++	+++	+++
		9	+++	+++	+++	+++	+
	Agammaglobulinemic patients	E. S.	++	+	+++	+++	−
		W. A.	++	+	++	++	−
		L. L.	++++	+	+++	++++	−
		F. H.	+++	+	+++	+++	−
Secondary response 4 days after second experience with antigen	Normal children	6	++++	+++	++++	++++	+++
		7	++++	++++	++++	++++	++++
		10	++++	+++	++	++++	+++
		11	++++	++++	++++	++++	++++
	Agammaglobulinemic patients	E. S.	++	++	+++	++++	−
		W. A.	++	++	++++	+++	−
		F. H.	++++	+	+++	++++	−
		L. L.	++++	++	++	+++	−

In Fig. 5, the structure of lymph nodes removed following antigenic stimulation of a normal child and a patient with agammaglobulinemia is compared. The greater thickness of the cortex and the greater germinal activity of the nodes from the normal patients is apparent.

In Fig. 6, medium-power views of the medullary portion of the nodes taken following antigenic stimulation from a normal subject and one with agammaglobulinemia are compared. Imprint preparations of comparable areas from representatives of the two groups are illustrated in Fig. 7.

The occurrence of clusters of plasma cells in the medullary cords of the lymph nodes from the normal persons during the period of antibody production

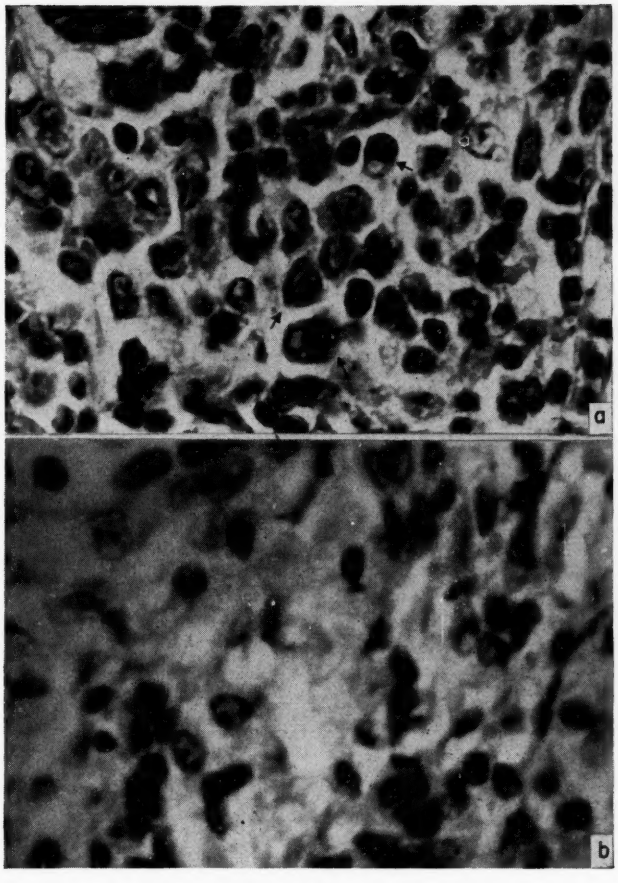

Fig. 6.—Comparison of the medullary portion of the lymph node from a normal child (*a*) and a patient with agammaglobulinemia (*b*) taken four days after antigenic stimulation with T.A.B. antigen. Note the abundance of plasma cells occurring in clusters in the lymph node of the normal child following antigenic stimulation and their complete absence from the nodes of the patient with agammaglobulinemia following identical antigenic exposure. (×1,000; reduced ½.)

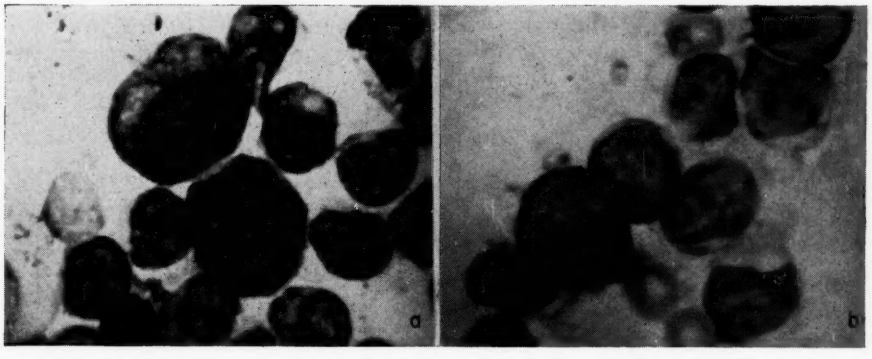

Fig. 7.—Comparison of imprints from the nodes of a normal child (*a*) and a patient with agammaglobulinemia (*b*) illustrating the morphologic features of the cells in the medullary cord of each following antigenic stimulation. Note the plasma cells and developing plasma cells in the node from the normal child and their absence from the patient with agammaglobulinemia. (×2,000; reduced ½.)

stands in striking contrast to complete absence of these cells from comparable areas in children with agammaglobulinemia. In Fig. 8 is illustrated the abundant plasmacytosis which features the morphologic alteration of lymph nodes of normal subjects during the secondary response to antigenic stimulation.

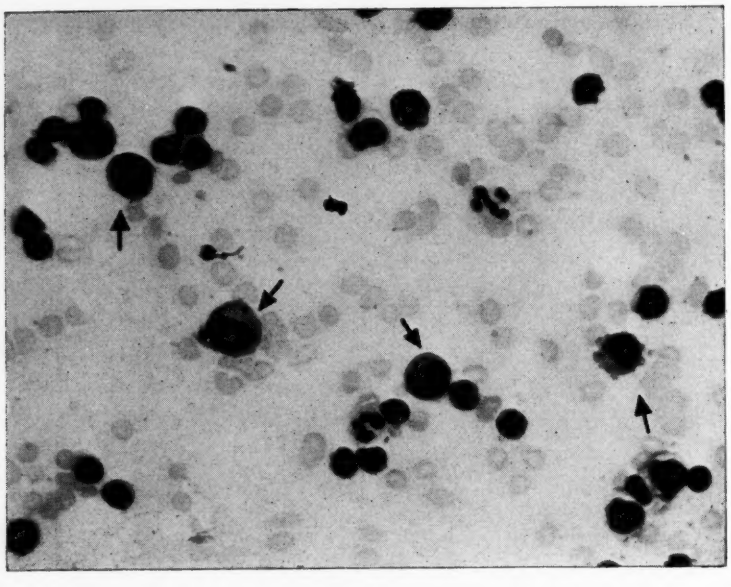

Fig. 8.—Medium-power view of the lymph node taken from the inguinal region of a normal child four days after a second injection of T.A.B. organisms (secondary response). Note the abundance of plasma cells in the imprint.

Whereas approximately one hundred plasma cells may be found in a single cross section of a lymph node from a normal child during the course of the primary response, several hundred and sometimes thousands of these cells and their precursors appear in the medullary portion of the node undergoing the secondary response. This intensity of plasma cellular proliferation parallels the greater speed and intensity of antibody production which characterizes the secondary response to antigen. In contrast to the intense plasma cell response in normal subjects, those with agammaglobulinemia failed to develop plasmacytosis as a consequence of even repeated exposure to antigen.

DISCUSSION

The observations herein reported must be interpreted as support for the concept that plasma cells are directly involved in the sequence of events resulting in the formation of antibodies and gamma globulin and the liberation of these proteins into the circulating blood. Along with evidence already available in the literature, some of which would appear to demonstrate directly and conclusively that plasma cells form antibodies in experimental animals, these findings make it tempting to conclude that plasma cells produce circulating antibody in man. As a corollary to this conclusion must be the acceptance of the concept that reflected in the morphologic features by which we identify plasma cells is

machinery essential to the production of gamma globulin and antibodies. Indeed, it was pointed out long ago by Downey[28] that plasma cells possess features common to many secretory cells. More recently, in studying the nucleic acid distribution in plasma cells, Thorell[29, 30] derived a similar conclusion. The cytoplasmic basophilia so characteristic of plasma cells is known to reflect high concentrations of ribonucleoprotein which has been linked by Dempsey and associates[31] and by others[32] to protein synthesis. The nucleus of this cell type has structural characteristics of cells actively engaged in a metabolic function or vigorously involved in self-reproduction. Further, the eccentric position of the nucleus, pushed off to the side as it were, is a feature common to many secretory cellular elements. Finally, the perinuclear clear area or so-called Hof, a distinguishing feature of plasma cells, appears, on phase microscopy and after special staining, to contain many mitochondria and a striking Golgi apparatus[33] which would be compatible with the concept that these cells are engaged in a secretory function.

Although attributing antibody production to plasma cells or to any single cell type undoubtedly represents an oversimplification of the morphologic foundation for a complex physiologic process involving several different types of cells, the recognition that a particular morphologic element plays an essential role in this important activity is of real significance. From perusal of the extensive clinical and experimental literature describing diseases, for example, erythroblastosis, rheumatic fever, hematologic disorders, and fibrinoid diseases, which seem to have as their basis mistaken or misdirected antibody formation and which now suggest that homotransplantation failure has an immunologic basis,[33-35] it becomes clear that ultimate control of antibody production will carry overwhelming rewards. To have a particular cellular objective in the quest for knowledge which we anticipate will ultimately provide the basis for such control is a real advantage. These data strengthen our contention, expressed earlier,[36] that detailed study of plasma cells, their functions, and requirements may be an incisive approach toward the understanding and ultimate control of the immune response.

SUMMARY AND CONCLUSIONS

1. Bone marrow and lymph nodes from four patients with agammaglobulinemia have been compared to similar tissues from normal children and adults. A deficiency of plasma cells in the tissues of the agammaglobulinemic patients is described.

2. Development of plasmacytosis of the bone marrow and regional lymph nodes of normal children following antigenic stimulation was observed.

3. That plasmacytosis of the draining node in normal children is quantitatively greater during the anamnestic reaction than during the primary response to antigen is indicated.

4. That lymph nodes of patients with agammaglobulinemia tend to be small, poorly developed, and have a relatively thin cortex with few germinal centers is pointed out.

5. Evidence indicating that lymph nodes and bone marrows of patients with agammaglobulinemia are characterized by failure to develop plasma cells and secondary follicles in response to both primary and secondary antigenic stimulation is presented.

6. The bearing of these observations on the cellular basis of the immune response is discussed.

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METABOLISM OF LANTHANUM AND YTTRIUM CHELATES

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INFORMATION is rapidly accumulating on the influence of chelating agents upon the fate of stable and radioactive metals in man. This development stems from attempts to selectively remove or localize certain metals or their respective compounds and thereby achieve therapeutic effects. The mobilization of calcium, iron, lead, yttrium, strontium, and plutonium has been studied in animals and in man.[1-5] Favorable therapeutic results have been reported in cases of lead poisoning.[3] The effect of ethylenediaminetetraacetic acid (EDTA) upon enzyme reactions in vitro,[6] upon blood clotting,[7] and upon calcium homeostasis[1] has been investigated. The influence of the Ca-EDTA chelate upon calcium exchange in man[8] has been studied.

While the pharmacologic action of chelating agents is of practical clinical importance, their use as a tool for the investigation of metal metabolism is of equal interest. Thus, differences in the metabolism of lanthanum were observed in this laboratory when the metal was injected in ionic and chelated form into normal and tumor-bearing mice and into human subjects.[9-11] From these observations it was inferred that the mammalian organism is capable of dissociating injected La-EDTA chelate which forms a very tight complex in vitro. The in vivo behavior of complex-bound lanthanum and yttrium was therefore investigated, employing two chelating agents of different binding characteristics, namely, EDTA which forms stable chelates with La and Y, and nitrilotriacetic acid (NTA) which forms somewhat less stable chelates with these metals.[12] By studying the fate of these metal complexes, it was hoped to obtain information on some of the factors affecting the metabolism of metals in the mammalian organism.

MATERIALS AND METHODS

Irradiated lanthanum or yttrium oxide† was dissolved in dilute hydrochloric acid as previously described.[13] La-EDTA, Y-EDTA, and Y-NTA were prepared by mixing solutions of lanthanum or yttrium chloride with solutions of the sodium salts of EDTA or NTA and subsequently adjusting the pH with NaOH to approximately 7. In order to ensure maximum chelation of the metal ions a fourfold excess of the chelating agent over the stoichiometric requirements (1:1 Molar Ratio) was used. All solutions were autoclaved before injection.

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†Irradiated units of La_2O_3 and Y_2O_3 containing La^{140} and Y^{90}, respectively, were obtained from Brookhaven National Laboratories.

These solutions were radio-assayed as previously described.[13] Doses of 20 to 120 μc were used for metabolic investigations, and of 175 to 500 μc for tissue distribution in human beings. The stable lanthanum or yttrium content ranged from 0.5 to 10 mg. per dose.

The metabolism of these metal chelates was studied in 14 cancer patients and was compared with that of ionic lanthanum or yttrium. In order to minimize biologic variations, the lanthanum or yttrium compounds were administered successively to the same patient. An intravenous infusion of 5 per cent glucose in water was started, and the radioactive material was injected into the tubing of the infusion set. In some cases, 10 ml. of a 20 per cent solution of Ca-EDTA was given as a single intravenous injection prior to the administration of the tracer doses of La^{140}- or Y^{90}- EDTA. In other studies, infusions of 500 ml. of 5 per cent glucose in water containing 4 to 8 grams Ca-EDTA were started immediately before the injection of the metal chelate and were continued for several hours. The infusions were given in the fasting state and food was withheld for four hours. During this time frequent blood samples were taken and urine was collected at corresponding intervals through an indwelling catheter. Subsequent blood specimens and urine collections were obtained at longer intervals. Stool analyses were performed in several experiments. In order to test reproducibility of the results, repeat studies were performed in some instances in the same subject.

Yttrium compounds were also injected intravenously into terminal cancer patients and analyses were performed on tissue samples obtained at autopsy of 4 cases. Distribution studies were also carried out on tissues of three groups of Strain A mice which had been injected intravenously with YCl_3 and the two Y-chelates, respectively. The animals were sacrificed forty hours after injection.

Radio-assays of La^{140} on blood and urine were carried out by gamma counting in Marinelli beakers. In counting Y^{90} an end-window tube was employed and the samples placed in planchets. One milliliter liquid samples were dried under an infrared lamp. Soft tissue samples were spread evenly on the planchets and dried overnight in an oven at 120° C. Bone samples were prepared by ashing at 500° C. overnight. No significant self-absorption was observed for dried samples of plasma and urine. The self-absorption in dried tissue and ashed bone samples (wet weight less than 1 gram) was observed to be less than 8 per cent.

An experimental standard was counted along with the samples and the results were expressed in per cent of the standard. Uranium standards were used to verify the stability of the counting equipment throughout the investigation. Decay curves indicated small amounts of a radiochemical impurity of long life in the irradiated units of Y_2O_3 which was first detectable about twelve days following removal from the reactor.

The statistical counting error varied. In the initial phases of the experiment, sufficient counts were taken to keep the probable error within 1.0 per cent. In later phases, counting rates were lower, and it was not feasible to maintain this precision, so that counting errors as high as 6 per cent were incurred. In distribution studies on human tissues, the counting rates of the least active samples were not much greater than two to three times background. For these low activity samples, the statisical counting error was as high as 10 per cent and therefore indicated values represent estimates.

OBSERVATIONS AND RESULTS

When $LaCl_3$ is administered intravenously, less than 5 per cent of the lanthanum is excreted by the kidneys in forty-eight hours, at which time the excretion has become negligible. When La-EDTA is administered, approximately 10 per cent of lanthanum is excreted in the urine in the same period of time. After the excretion has declined to negligible amounts, an infusion of 4 Gm. Ca-EDTA in 500 ml. of 5 per cent glucose in water, given intravenously

over four hours, promoted additional urinary excretion of lanthanum. Excess Ca-EDTA was most effective in promoting urinary excretion of La^{140} when given simultaneously with, or very soon after, the administration of the radioisotope, resulting in a cumulative excretion of approximately 40 per cent.[10, 11] The rates of excretion following the injection of the lanthanum compounds are shown in Fig. 1.

Plasma levels following the injection of $LaCl_3$, and La-EDTA in the presence of excess Ca-EDTA were measured. It was found that the plasma retention of lanthanum injected as the chloride is greater than that of the EDTA chelate.

Similar studies were carried out with yttrium. The general chemical properties resemble those of lanthanum but it forms chelates of greater stability with EDTA and NTA.[12] A representative experiment illustrating differences in urinary excretion of yttrium following the injection of the various yttrium compounds, is shown in Fig. 2. It was found that Y^{90} was excreted least after the injection of YCl_3, while the greatest excretion occurred when Y^{90}-EDTA was

TABLE I. TRANSFER OF YTTRIUM FROM PLASMA INTO EXTRAVASCULAR SITES

	TIME (MINUTES)	PLASMA CONTENT (PER CENT)	CUMULATIVE URINARY EXCRETION (PER CENT)	CHANGE IN CUMULATIVE URINARY EXCRETION IN PER CENT (ΔU)	CHANGE IN PLASMA CONTENT IN PER CENT (ΔP)	ΔP-ΔU (PER CENT)	ΔT (MINUTES)	$\frac{\Delta P - \Delta U}{\Delta T}$ (% PER MINUTE)
	0	—	—	—	—	—	—	—
	10	28.7	—	—	—	—	—	—
	25	20.2	9.7	9.7	79.8	70.1	25	2.80
	40	16.3	—	—	—	—	—	—
	60	14.1	23.3	13.6	6.1	-7.5	35	-0.21
	80	11.9	28.6	5.3	2.2	-3.1	20	-0.16
	100	10.4	—	—	—	—	—	—
	120	7.8	35.5	6.9	4.1	-2.8	40	-0.07
Y^{90}EDTA	140	6.4	40.4	4.9	1.4	-3.5	20	-0.18
+	160	6.4	44.5	4.1	0.0	-4.1	20	-0.21
Ca EDTA	180	5.7	48.1	3.6	0.7	-2.9	20	-0.15
	240	4.5	57.1	9.0	1.2	-7.8	60	-0.13
	480	1.8	71.9	14.8	2.7	-12.1	240	-0.05
	720	0.7	77.7	5.8	1.1	-4.7	240	-0.02
	1,440	0.0	80.6	2.9	0.7	-2.2	720	-0.003
	0	—	—	—	—	—	—	—
	10	95.2	—	—	—	—	—	—
	25	79.1	0.058	0.058	20.9	20.8	25	0.83
	40	71.0	0.099	0.041	8.1	8.1	15	0.54
	60	62.6	0.153	0.054	8.4	8.3	20	0.42
	80	57.8	0.181	0.028	4.8	4.8	20	0.24
$Y^{90}Cl_3$	100	53.0	0.210	0.029	4.8	4.8	20	0.24
	120	49.8	0.244	0.034	3.2	3.2	20	0.16
	140	44.9	0.262	0.018	4.9	4.9	20	0.25
	160	42.9	0.288	0.026	2.0	2.0	20	0.10
	180	41.2	0.314	0.026	1.7	1.7	20	0.09
	240	36.6	0.369	0.055	4.6	4.5	60	0.08
	480	22.7	0.659	0.290	13.9	13.6	240	0.06
	720	15.3	0.809	0.150	7.4	7.2	240	0.03
	1,440	5.8	1.569	0.760	9.5	8.7	720	0.012

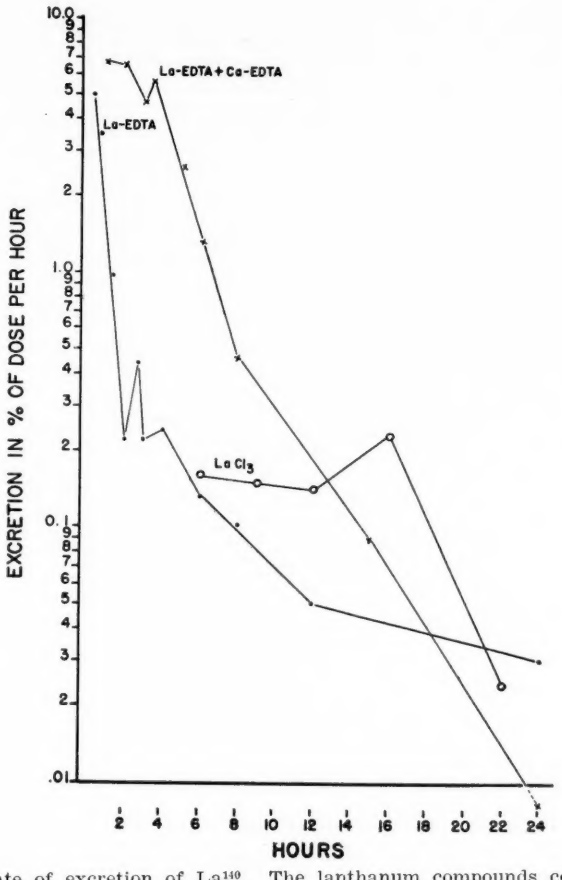

Fig. 1.—Rate of excretion of La^{140}. The lanthanum compounds containing tracer doses of La^{140} were administered intravenously. The molar ratio of LA to EDTA was approximately 1:1. In the study, indicated by x——x, 1 Gm. Ca-EDTA was injected intravenously and was followed immediately by a 4-hour infusion of 5 per cent glucose in water containing La-EDTA (145 μc La^{140}, 1.5 mg. La^{+++}) and Ca-EDTA (4 Gm.).

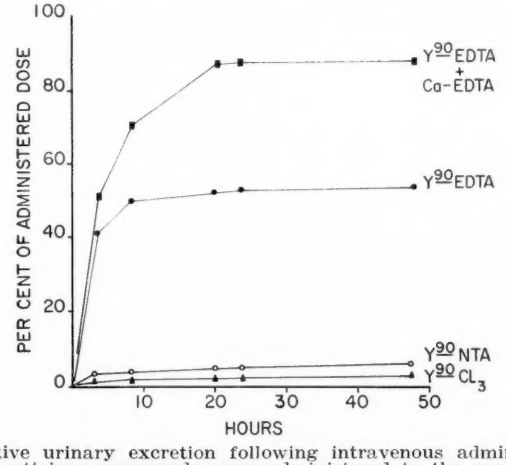

Fig. 2.—Cumulative urinary excretion following intravenous administration of yttrium^{90} compounds. The four yttrium compounds were administered to the same patient: Y^{90}-EDTA + Ca-EDTA (a single intravenous injection of 2 Gm. Ca-EDTA was immediately followed by the injection of a tracer dose of Y^{90}-EDTA, Y^{+++} content <1.0 mg.). This was followed by the injection of Y^{90}-EDTA, Y^{90}-NTA, $Y^{90}Cl_3$, at 48-hour intervals.

injected in the presence of excess Ca-EDTA. Although the absolute amounts excreted varied from patient to patient, the relative excretion levels of the different compounds were similar.

The Y^{90} plasma levels declined slowly following the injection of the chloride while they rapidly decreased following the administration of Y-NTA and Y-EDTA. For instance, ten minutes after the injection of YCl_3, approximately 95 per cent of the dose was still present in the estimated total plasma volume, whereas only 29 per cent of the dose could be accounted for in the plasma following the injection of Y-EDTA plus Ca-EDTA. At four hours, the values were approximately 37 and 4.5 per cent, respectively (Table I). The plasma levels expressed as per cent of dose per milliliter are shown in Fig. 3.

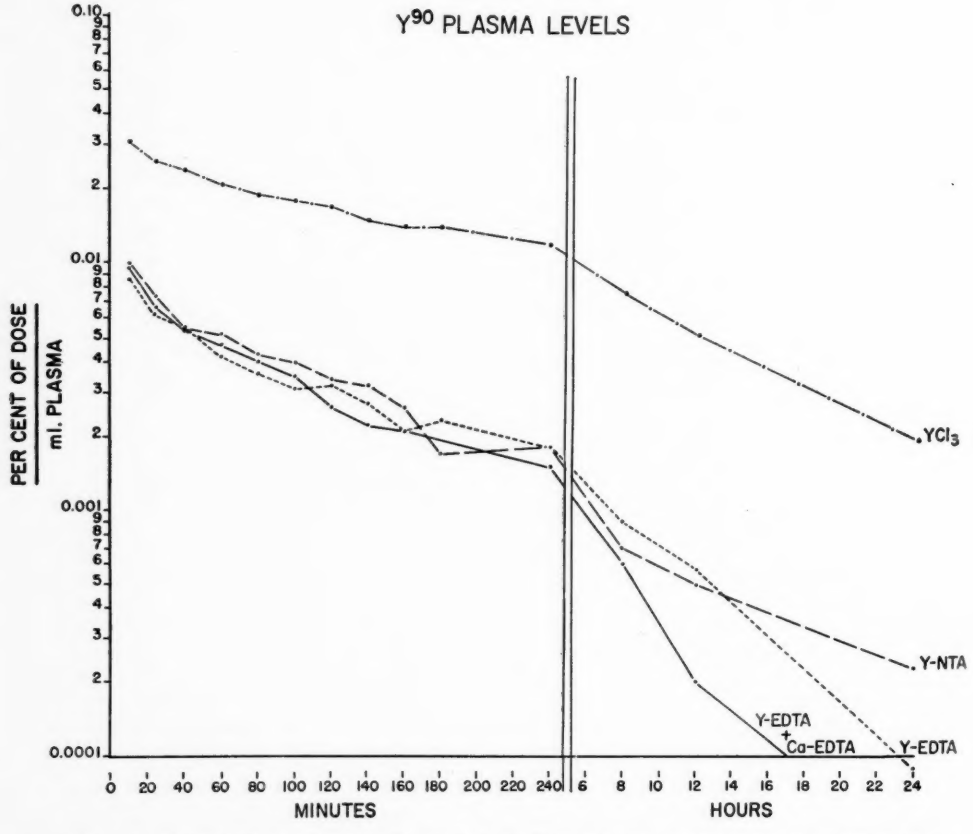

Fig. 3.—The yttrium compounds were administered to Patient L. D.; Y^{90}-EDTA + Ca-EDTA (a single intravenous injection of 2 Gm. Ca-EDTA was immediately followed by the injection of a tracer dose of Y^{90}-EDTA, Y^{+++} content <0.5 mg.). This was followed by the injection of Y^{90}-EDTA, Y^{90}-NTA, Y^{90}-Cl_3 at 24-hour intervals.

The change in Y^{90} distribution as a function of time is further illustrated in Fig. 4, which shows the cumulative urinary excretion, the calculated plasma content, based upon estimated plasma volumes, and the amount unaccounted for and presumably present in the tissues. Yttrium increases in the tissues at the

expense of the yttrium content of the plasma following the injection of YCl_3 and of Y-NTA so that, after twenty-four hours, over 90 per cent is present in the tissues. After the injection of Y-EDTA, the yttrium content of the tissues declines from a twenty-minute value of 70 per cent to approximately 20 per cent at the end of twenty-four hours.

The ratio of the cumulative excretion of YCl_3 to Y-EDTA in the presence of excess EDTA is of the order of 1:15. To further analyze the differences observed in the urinary excretion of the various yttrium compounds, the ratio of the yttrium plasma level to the urinary excretion rate was calculated (Fig. 5). It is seen that the kidneys are several hundred times less effective in removing yttrium injected as the chloride than as the EDTA in the presence of excess Ca-EDTA.*

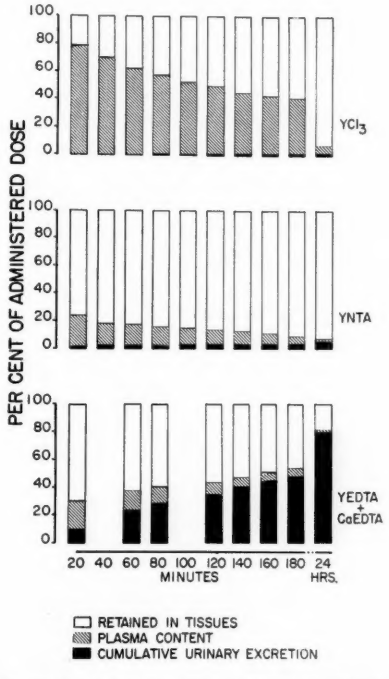

Fig. 4.—See legend of Fig. 3, Patient L. D. The plasma content was calculated on the basis of an estimated total plasma volume of 3,000 ml.

The derived quantity, $\frac{\Delta P - \Delta U}{\Delta t}$ has been calculated for the compounds YCl_3 and Y-EDTA plus Ca-EDTA (Table I and Fig. 6). ΔP is the calculated change in plasma content in the time interval Δt, and ΔU is the urinary excretion during the same period. An interpretation of the graphs in terms of net transfer rates is given below.

In order to ascertain the sites of deposition of yttrium following its intravenous administration, tissue distribution studies have been carried out in Strain A mice. The results are given in Table II. Following the injection of

*Excess Ca-EDTA was added to suppress dissociation of the yttrium chelate.

YCl_3, 77 per cent of the yttrium was retained in the tissues, principally in the liver. The greatest concentration of yttrium following the injection of Y-NTA occurred in bone, liver, kidneys, and spleen. Minimal retention was observed following administration of Y-EDTA. Tissue distribution studies were also carried out on autopsy specimens obtained from four terminal cancer patients

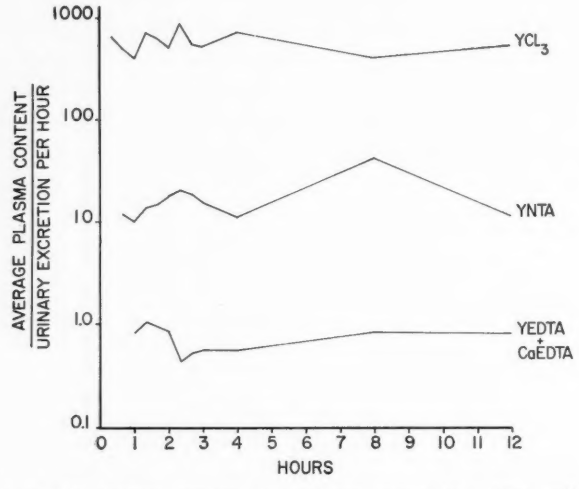

Fig. 5.—Ratio of Y^{90} plasma content to urinary excretion. The ratios were calculated on the basis of Y^{90} plasma content and Y^{90} urinary excretion of Patient L. D. (See Figs. 3 and 4.)

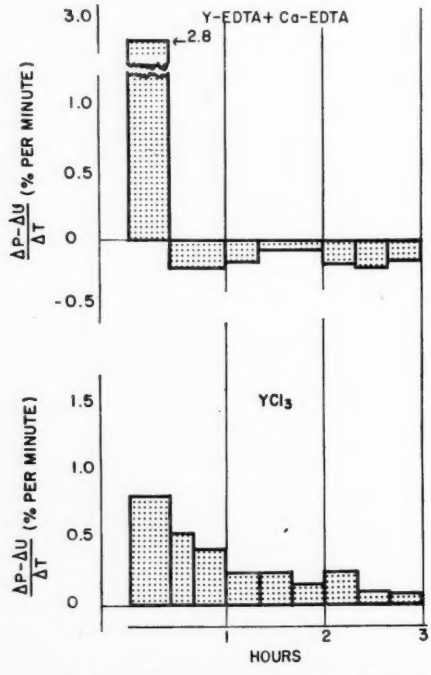

Fig. 6.—Transfer of yttrium from plasma to extravascular sites. See last column, Table I. (Patient L. D.)

TABLE II. DISTRIBUTION OF Y^{90} IN MICE

TISSUE	$Y^{90}Cl_3$		Y^{90} NTA		Y^{90} EDTA	
	PER CENT OF INJECTED DOSE PER		PER CENT OF INJECTED DOSE PER		PER CENT OF INJECTED DOSE PER	
	ORGAN	GM. TISSUE	ORGAN	GM. TISSUE	ORGAN	GM. TISSUE
Muscle	1.46	0.13	7.50	0.70	0.15	0.01
Bone	1.86	1.17	8.40	5.30	0.64	0.38
Lung	0.67	3.3	0.04	0.23	0.005	0.21
Heart	0.07	0.41	0.01	0.07	0.002	0.008
Liver	69.80	46.10	5.80	4.70	0.09	0.06
Spleen	0.07	0.25	0.55	2.47	0.003	0.01
Kidneys	0.42	1.39	0.94	3.16	0.05	0.12
Gastrointestinal tract	2.52	0.63	3.30	1.30	0.08	0.02
Total	77		27		1.0	

The animals were sacrificed 40 to 45 hours after the intravenous injection of Y^{90}. 26 μc Y^{90} and 0.19 mg. Y^{+++} were injected as the chloride and as the NTA-chelate: 17 μc Y^{90} and 0.13 mg. Y^{+++} were injected as the EDTA-chelate. The values listed in the table represent average tissues from 3 mice.

who had been injected intravenously with yttrium compounds. The results are summarized in Table III. Although the amounts of yttrium present per gram of human tissue are much smaller than in the mouse (Table II) the fractions per organ are of the same order of magnitude.

TABLE III. TISSUE DISTRIBUTION OF Y^{90} IN MAN

COMPOUND ADMINISTERED	HOURS*	MUSCLE		LIVER		BONE SAMPLES		
						VERTEBRA	RIB	FEMUR
		PER CENT ORGAN†	PER CENT GM.	PER CENT ORGAN†	PER CENT GM.	PER CENT GM.	PER CENT GM.	PER CENT GM.
Y-NTA	7	13	0.0005	21	0.016	0.017	0.011	0.0007
Y-NTA	14	14	0.0006	30	0.025	0.009	0.009	
Y-NTA	216	9	0.0004	8	0.006	0.016	0.010	0.003
Y-EDTA	28	9	0.0004		0.0013 0.0024‡	0.005	0.005	

The compounds were administered intravenously. The values are expressed in per cent of the injected dose.

*Interval between injection and death.

†Based upon total organ weight.

‡Liver tissue replaced by tumor.

DISCUSSION

When $LaCl_3$ is injected intravenously, the urinary excretion of lanthanum is low and the plasma levels remain high. This suggests that high molecular weight complexes are formed, probably lanthanum proteinates, which neither diffuse rapidly into the extravascular compartment nor are readily excreted by the kidneys.

Since EDTA is not metabolized and is promptly excreted,[14, 15] one might expect that the excretion of the strong metal chelate La-EDTA would also be rapid. However, the total excretion of lanthanum when injected as La-EDTA is hardly more than 10 per cent of the amount injected. This raises the possibility that the metal chelate is subject to dissociation in the body. The effectiveness of excess EDTA in form of Ca-EDTA administered at the time of injection, in enhancing renal excretion[10, 11] might thus be due to simple mass action, that is, to a tendency to suppress the dissociation of the La-EDTA complex.

When excess Ca-EDTA is administered several days after the initial dose of La-EDTA, there is a sharp rise in the rate of renal excretion of lanthanum although the total increase in cumulative urinary excretion is small.[11] Thus, it would appear that several days after the injection most of the lanthanum is either physically or chemically inaccessible and therefore the effectiveness of excess EDTA in mobilizing the radioisotope as the readily excretable La-EDTA complex is reduced.

Since Y-EDTA is more stable than La-EDTA, less dissociation and, therefore, more excretion of Y^{90} would be expected and was, in fact, observed. On the other hand, the excretion of Y-NTA, a less stable metal chelate, was small and comparable to that of La-EDTA.

Excess EDTA is more effective in mobilizing yttrium than lanthanum, and when large amounts of excess EDTA are administered close to the time of injection, the urinary excretion of yttrium ranges from 80 to 90 per cent of the injected dose. These results are in agreement with the findings of Cohn and associates,[4] who observed that Na-EDTA and Ca-EDTA are effective agents in enhancing the urinary excretion of yttrium in rats. They also showed that the effectiveness of EDTA in mobilizing yttrium depends upon the time interval between the administration of the metal isotope and the chelate which is in keeping with the observations made by us on lanthanum.[11]

The similarity of plasma levels following injection of yttrium chelates can be seen in Fig. 3, while the dissimilarity of their metabolism is shown in Fig. 4. It is seen that while the yttrium chelates diffuse at comparable rates from the vascular to the extravascular compartment, yttrium administered in the form of the NTA-chelate is ultimately retained in the tissues, whereas yttrium injected as Y-EDTA readily re-enters the vascular system and is then very efficiently excreted.

In an attempt to determine the rate at which the net transfers of yttrium administered as the chloride or the EDTA-chelate occurred between the vascular and extravascular compartments, the quotient $\frac{\Delta P - \Delta U}{\Delta t}$ was calculated for the first three hours (Table I). Fig. 6 shows that this quotient is negative for the yttrium chelate after the first twenty-minute period. The negative quotient indicates a net return flow of yttrium from the extravascular sites into the vascular compartment.

Foreman and co-workers have shown in rats[14] and man[15] that C^{14}-labeled Ca-EDTA diffuses readily from the vascular compartment into the tissues and that approximately 98 per cent is excreted in the urine within twenty-four hours. Since the urinary excretion of lanthanum and of yttrium chelates in the present experiments was much smaller, these metal chelates were either retained as such or, in part, dissociated, the chelating agent being excreted, and the liberated metals being substantially retained. The mechanism of this retention of rare earths by the organism as compared to the prompt clearance of alkaline earth chelates (Ca-EDTA) is as yet obscure. It should be noted, however, that no information is available concerning the handling of NTA by the organism. Studies on its metabolic fate will have to await the synthesis of C^{14}-labeled NTA.

The results here reported demonstrate that the excretion and tissue distribution of certain isotopes within the mammalian organism can be experimentally modified by means of complexing agents. Preferential concentration of radiometals in certain tissues or uniform distribution in the organism may thus be attained. The *strength* of the metal-chelate bond is only one of the factors determining the rate of excretion and the tissue distribution of metals. The *rate* of metal exchange reactions as a function of the chelating agent and the environment is no doubt of equal importance. In the instance of EDTA, for example, calcium ions tend to equilibrate much more rapidly with the metal chelate and the chelating agent in solution than does iron.[16] Furthermore, the amount of metal ion in free form varies greatly as a function of the amount of excess chelating agent. Thus, a tenfold increase in the concentration of EDTA at pH 7.4 will decrease the concentration of Ni^{++}-ions tenfold over the range from 10 per cent to 100 per cent molar excess of chelating agent.[17]

SUMMARY

The metabolism of lanthanum and yttrium administered intravenously in ionized form or as chelates of ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) to human subjects and experimental animals was further studied. La^{140} and Y^{90} were used as tracers. It was found that the plasma content, urinary excretion, and tissue uptake of these metals depended upon the compound administered.

Following the intravenous injection of La and of Y in ionized form, high and sustained plasma levels, high uptake by the liver, and low urinary excretion were observed. It is assumed that these metals form high molecular complexes, possibly with the plasma proteins, which are not excreted by the kidney and were transferred to and mainly deposited in the liver.

Following the intravenous injection of metal chelates, the cumulative urinary excretion ranged from < 5 per cent to > 85 per cent; La^{140}-EDTA and Y^{90}-NTA were excreted least, while Y^{90}-EDTA in the presence of excess Ca-EDTA was excreted most.

Yttrium injected as the NTA-chelate also disappeared rapidly from the blood stream although the urinary excretion was low. There was no evidence of net re-entry into the vascular compartment, and yttrium was localized in various tissues with areas of concentration in bone, liver, spleen, and kidney.

The forced removal and the modification of distribution of radioisotopes for the purpose of obtaining uniform distribution or preferential concentration in certain tissues by the use of chelating agents have been discussed.

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URINARY EXCRETION OF ACID MUCOPOLYSACCHARIDES BY DIABETIC PATIENTS

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INTRODUCTION

IN RECENT years considerable interest has been focused upon the vascular and renal complications of diabetes which have become increasingly apparent with the use of insulin. Indications are that the complicating lesions reflect the abnormal metabolism of polysaccharides. Both the hyaline masses in intercapillary glomerulosclerosis and the thickened walls of retinal capillary aneurysms have been shown repeatedly to stain by the periodic acid-Schiff method, thus demonstrating the presence of polysaccharides and/or certain unsaturated lipids or phospholipids. On this PAS staining reaction, Gilliland and associates[1] based their initial assumption that protein-bound polysaccharides are present in the hyalinized material of the arterioles of both the renal and retinal lesions of diabetes. They then demonstrated by paper electrophoretic means that there is a progressive rise in the total serum protein-bound polysaccharides as diabetic complications ensue, evident before clinical renal complications are demonstrable. The over-all findings were interpreted as supporting the suggestion made by several investigators[2-6] that the source of the hyaline material deposited in the glomeruli and elsewhere may be a polysaccharide present in the blood of the diabetic patient in abnormal amounts. Warren and LeCompte[7] have indeed suggested that most of the characteristic changes in diabetes mellitus may be interpreted in terms of a disturbance in the metabolism (or structural organization) of a simple polysaccharide (glycogen) or of more complex polysaccharides (mucopolysaccharides).

In view of the suggestions offered by these reports, it was thought that useful information might be obtained by a quantitative study of the urinary acid mucopolysaccharides in diabetes with vascular and renal complications. An attempt was also made to determine whether or not there was any abnormality in response to the oral administration of glucuronolactone, a pro-

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cedure which has been shown to cause, in normal subjects, a marked increase in the urinary excretion of substances containing hexuronic acid and precipitating with the acid mucopolysaccharides.[8*]

METHODS

Subjects were divided into groups as follows. In Group 1 were laboratory personnel and nondiabetic patients, white and Negro, without clinical evidence of nephropathy, ranging in age from 23 to 70 years.† Group 2 included: (a) diabetic patients without evidence of renal impairment or of diabetic retinopathy; (b) diabetic patients without evidence of nephropathy but with diabetic retinopathy (minimal but definite retinal vascular changes including capillary aneurysms were present in 3 patients; marked diabetic retinopathy was present in one patient, WF58); and (c) diabetic patients with diabetic retinopathy and evidence of significantly impaired renal function, (significant and persistent proteinuria was present in all patients in this group; phenolsulfonphthalein excretions ranged from 15 to 55 per cent in two hours in the group; with one exception [WM23], diastolic blood pressures were 110 or higher). Group 3 contained nondiabetic patients with evidence of significant renal disease (some degree of proteinuria was present in all patients; phenolsulfonphthalein excretion ranged from 6 to 35 per cent in two hours in the group). Base-line 24-hour urines were collected for 1 to 7 days, following which, in some instances, another series of 24-hour collections was made with the patient receiving 5 Gm. of glucuronolactone each day. During the entire period of observations, medications which might interfere with interpretation of the results were withheld. Acid mucopolysaccharides were isolated from the urine by a modification of the method of Astrup.[10‡] The hexuronic acid content of the Astrup fraction was determined by the carbazole method of Dische.[11] Tubes were set up and hydrolyzed in quadruplicate, one tube later receiving

*Acid mucopolysaccharides contain both hexuronic acid and hexosamine. Studies of hexosamine excretion in this laboratory subsequent to a previous publication[8] have shown a marked discrepancy in the degree of rise of the hexosamine component of the Astrup fraction, following the ingestion of glucuronolactone, as compared to the rise in the hexuronic acid component. This is due at least in part to the excretion of a dialyzable substance containing hexuronic acid which is precipitable with the acid mucopolysaccharide fraction. Chondroitin sulfate is not dialyzable. The dialyzable substance has not been identified at this time. Examples of the data referred to are as follows, using for analysis Astrup fractions of nondialyzed and dialyzed urine collected for 3 hours under standard conditions from 4 normal subects, with and without prior ingestion of glucuronolactone (GL):

	EXPRESSED AS MG. GLUCURONOLACTONE		EXPRESSED AS MG. N-ACETYL GLUCOSAMINE	
	NONDIALYZED	DIALYZED	NONDIALYZED	DIALYZED
Base line ♂	0.983	0.715	1.846	1.516
after GL	2.450	0.746	1.920	1.246
Base line ♂	0.723	0.456	1.322	0.904
after GL	2.500	0.544	1.784	1.080
Base line ♀	0.504	0.321	0.946	0.656
after GL	4.539	0.834	1.348	0.860
Base line ♀	0.534	0.269	0.714	0.576
after GL	7.811	0.313	1.698	0.548
Commercial chondroitin sulfate 0.200 mg.	0.039	0.042	0.055	0.056

On inspection of the base-line values after dialysis, an inequality in molecular amounts of glucuronolactone and n-acetyl glucosamine is noted. The nondialyzable material isolated from urine is not hydrolyzed by testicular hyaluronidase. In view of the observation of Meyer[9] that chondroitin sulfate B gives only one-half the carbazole value given by A and C, the nondialyzable material is being subjected at this time to purification procedures and further analysis to determine more exactly its structure.

†The new group of controls was made necessary by the minor change in technique (see later footnote) used in the present study as contrasted with that in Reference 8. Somewhat increased normal values were obtained using this modification of the previously reported method.

‡(a) The filtration step after separation with ether was omitted. (b) Three milliliters of aqueous mixed salt solution (17 per cent NaCl, 1.5 per cent Na_2SO_4) was added to 47 ml. aliquot of urine in each instance because precipitation of acid mucopolysaccharides was otherwise incomplete in specimens obtained from patients on certain salt-restrictive diets.

0.2 ml. of alcohol alone to serve as a color control. Standard curves employing two tubes each of 20, 30, and 40 gamma amounts of glucuronolactone were run simultaneously with each set of determinations, using a Coleman Junior spectrophotometer for readings. Results were discarded if the highest and lowest readings of any set of tubes failed to check within 15 per cent of each other, and the entire run was discarded if duplicate readings on the standard curve failed to check or if the standard curve did not pass within 0.035 of the zero point.

RESULTS

Results of base-line studies are presented in Table I. Fig. 1 illustrates graphically the course of excretion of the Astrup fraction by a few of the subjects.

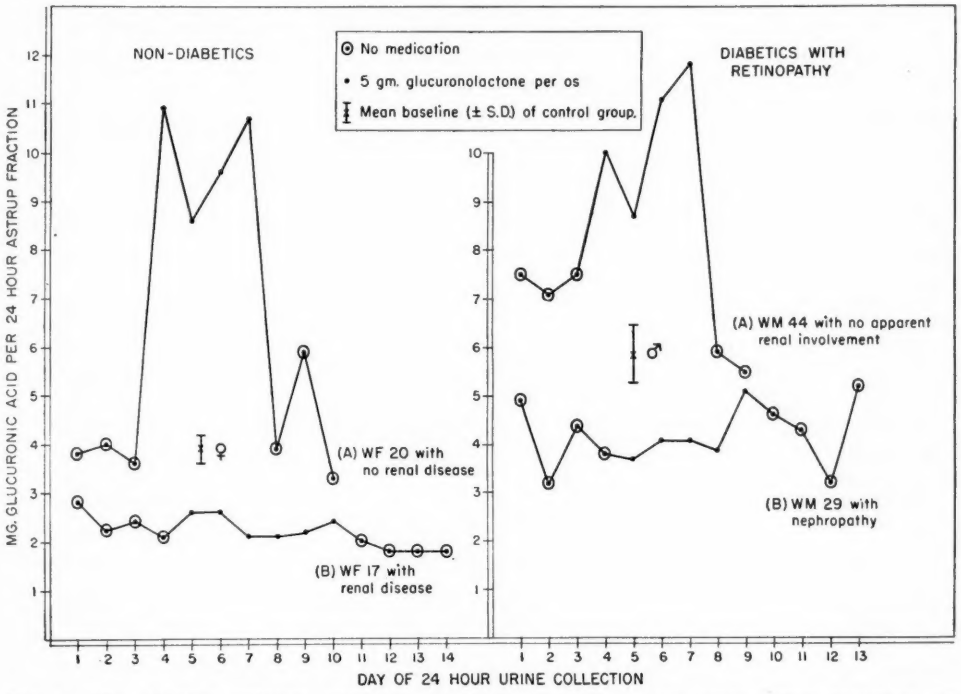
NON-DIABETICS
No medication
5 gm. glucuronolactone per os
Mean baseline (± S.D.) of control group.
DIABETICS WITH RETINOPATHY
MG. GLUCURONIC ACID PER 24 HOUR ASTRUP FRACTION
(A) WF 20 with no renal disease
(B) WF 17 with renal disease
(A) WM 44 with no apparent renal involvement
(B) WM 29 with nephropathy
DAY OF 24 HOUR URINE COLLECTION

Fig. 1.—The 24-hour urinary excretion of Astrup fraction (see text) after ingestion of glucuronolactone.

It will be noted (Table I) that the base-line excretion of Astrup fraction in diabetics with normal renal function was slightly, but significantly, higher ($p < 0.01$) than that of nondiabetic individuals.* More recent studies have shown that most of this elevation is due to a dialyzable component which must be presumed not to be chondroitin sulfate. With impairment of renal function, the base-line excretion by diabetics was inconsistent as compared to that of controls, ranging from below to above normal in value.† Nondiabetics with severe renal disease were consistently low in their base-line excretions, however.

*Only those patients in Groups 2, (a) and (b) in whom 3 or more base-line observations were available were used in the statistical comparison of values with those of the control group of Table I. Male and female groups were compared separately.

†It is of interest that the low excretion recorded for WM29 in Table I was noted in the presence of as high urinary sugars as were encountered in the present study.

TABLE I. TWENTY-FOUR-HOUR URINARY EXCRETION OF TOTAL ASTRUP FRACTIONS* BY HUMAN SUBJECTS, EXPRESSED IN MILLIGRAMS OF GLUCURONIC ACID, MEAN ± STANDARD DEVIATION

GROUP	MALES		FEMALES	
1. Controls	5.9 ± 0.6	(9 subjects)	3.9 ± 0.3	(10 subjects)
2. (a) Diabetics, uncomplicated	10.9 ± 1.3	WM51 (8)†	5.6 ± 0.5	WF63 (4)
	8.4 ± 2.5	WM52 (5)	4.9 ± 0.8	WF63 (5)
	6.5 ± 0.7	WM43 (4)	5.0 ± 0.7	CF51 (3)
			4.3 ± 0.9	CF55 (3)
(b) Diabetics with retinopathy	6.4 ± 0.7	WM66 (9)	5.3 ± 0.1	WF58 (3)
	9.1 and 8.7	WM53 (2)	8.9 and 10.2	CF59 (2)
	6.7 ± 0.9	WM44 (5)		
(c) Diabetics with retinopathy and nephropathy	5.1 and 5.4	WM23 (2)	5.7 and 5.8	WF61 (2)
	3.4 and 2.9	WM35 (2)	3.1 ± 0.9	CF50 (8)
	4.2 ± 0.7	WM29 (8)	7.5 ± 2.1	CF40 (3)
3. Nondiabetics with nephropathy	3.7 ± 0.5	WM76 (7)	2.1 ± 0.4	WF17 (8)
	2.5 ± 0.3	WM44 (7)	2.7 ± 0.6	WF35 (8)
	4.9 ± 1.1	WM47 (7)		

*See footnote to Introduction.

†Number in parentheses refers to the number of base-line 24-hour urine levels determined on each patient. From 3 to 7 base-line levels were run on each control subject, the average number being 5.

After the oral administration of glucuronolactone, diabetics showed the same increased excretion of hexuronic acid compounds in the Astrup fraction as was observed in normal individuals. With sufficient renal damage, both diabetics and nondiabetics failed to show such increased excretion (Fig. 1). Isolated exceptions to this trend occurred.

DISCUSSION

The material fractionated from urine (termed Astrup fraction; see Method) and measured in the present study contains materials which are probably properly designated as acid mucopolysaccharides. They are nondialyzable compounds containing hexuronic acid and hexosamine and exhibiting certain chromatographic similarities to chondroitin sulfate as described elsewhere.[8]* However, the fraction also contains some dialyzable hexuronide material which cannot be accepted as chondroitin sulfate (see footnote to Introduction). Such dialyzable material increases sharply in the Astrup fraction after the ingestion of glucuronolactone. In the presence of significant impairment of renal function, whether in diabetics or nondiabetics, the total amount of Astrup fraction excreted is decreased, and no increase in the dialyzable component occurs after the ingestion of glucuronolactone.

In comparing diabetics and nondiabetics, the only difference noted by the techniques of the present study was the presence in the Astrup fraction of base-line diabetic urines of a usually dialyzable hexuronide component which

*When they are subjected to the action of testicular hyaluronidase (Alidase, G. D. Searle & Company) or B-glucuronidase (Ketodase, Warner-Chilcott Laboratories), no hexosamine can be detected without additional acid hydrolysis.

was not identified and which bore no constant relationship to the degree of glycosuria. It differed in at least one respect from the similarly dialyzable component described in the Astrup fraction of the urine of normal human beings following the ingestion of glucuronolactone, in that the dialysate of such specimens from normal human beings showed an additional reducing spot chromatographically not seen in the Astrup fraction of the dialysate of baseline diabetic urines.

It is apparent that the present study leaves unanswered the question posed concerning the presence in the blood of certain diabetics of an abnormal amount of mucopolysaccharides perhaps related to the hyaline material deposited in arterioles of renal and retinal lesions. While this postulate is in no way denied by the data presented here, still it receives no support in terms of demonstration of abnormal amounts of such substances excreted in the urine. The questions concerning molecular size of such substances and the possibilities of glomerular filtration have already been discussed.[8] Suffice it to say here that some studies indicate that chondroitin sulfate may exist in a molecular size that would permit glomerular filtration. The possibility also exists, however, that filtration would not occur in a highly polymerized state but rather in the form of a smaller unit. Such a unit has never been conclusively demonstrated in blood or urine of human beings.

SUMMARY

1. Quantitative studies are presented, dealing with a fraction obtained from human urine which includes (a) a nondialyzable, acid mucopolysaccharide-like compound resembling chondroitin sulfate, and (b) a dialyzable hexuronide component not presumed to be chondroitin sulfate. The latter component normally rises sharply following the ingestion of glucuronolactone.

2. The total fraction excreted was quantitatively decreased in urines from patients with impaired renal function. The dialyzable component failed to show the customary rise in excretion following the ingestion of glucuronolactone by such patients. These changes were unrelated to diabetic state.

The total fraction excreted in the urine of diabetics without demonstrable renal disease was slightly, but significantly, greater than that of controls. No consistent increase in the excretion of nondialyzable chondroitin-sulfate-like material was demonstrated in the fraction. The increased values were usually due to the presence of an increased dialyzable component which is probably not the same as that which is excreted following the ingestion of glucuronolactone.

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EXPERIMENTAL AND STATISTICAL STUDIES ON Rh ANTIBODIES

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THERE are few fields of medical science in which progress has been so rapid and so extensive as in the field of erythroblastosis fetalis. Here, as in other fields, the problems seem to have become increasingly complicated the further our knowledge extends. It is obvious that the theories which were founded on the original observations are no longer adequate to explain the discrepancies which the intensive studies of recent years have revealed.

In this series of studies, work undertaken to determine the reason for some of these discrepancies is described. In the first section the accepted theory of the mode of action of blocking antibodies and alternative possibilities are discussed. The second section is devoted to experiments designed to determine the mode of action of blocking antibodies. This is followed by a section discussing the different forms of antibody revealed by statistical analysis of the results of these experiments. In a subsequent section, the results of various techniques for the estimation of Rh antibodies are interpreted in the light of these findings.

PART I

EVALUATION OF ALTERNATIVE THEORIES FOR THE MODE OF ACTION OF BLOCKING ANTIBODIES

In 1944, Wiener[1] demonstrated that, in addition to the saline or bivalent Rh agglutinin, there also existed a "blocking" or univalent antibody. This was confirmed in the same month by Race,[2] who called this second type of Rh antibody the "partial" or "incomplete" antibody. Further supporting evidence was published in 1945 by Diamond and Abelson[3] who called it an "inhibitor" substance because their attempts to pool sera of different anti-Rh specificity sometimes resulted in loss of anti-Rh_0 (anti-D) agglutinin.

Wiener[1] demonstrated the blocking effect of this antibody by the failure of potent anti-Rh agglutinating sera to agglutinate Rh-positive cells which had first been incubated with serum from mothers whose babies developed erythroblastosis. These sera did not contain saline (bivalent) Rh antibodies. The prior treatment of the cells with such sera had, he suggested, coated them with blocking antibody which had prevented subsequent agglutination. It is generally believed that the blocking antibody is preferentially adsorbed onto Rh-positive red blood cells placed in a mixture containing blocking and agglutinating antibodies. If, therefore, the concentration of blocking antibody is sufficiently high, no saline agglutination will occur.

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In 1945, Wiener[4] found that if Rh-positive cells, suspended in serum, plasma, or albumin (human or bovine) instead of the usual saline medium, were incubated with sera containing blocking antibody, agglutination of the red cells would take place. This conglutination technique provided a simpler method of testing for these "blocking" (univalent) antibodies.

We have been puzzled by repeated observations that many sera containing saline agglutinins in sufficient concentration to be used for routine Rh testing have also a very high albumin titer. According to the adsorption theory, one would expect the amount of blocking antibody present in such sera to be sufficient to completely block all of the cells so that no agglutinating effect should be obtained. We have, therefore, considered the possibility that blocking antibodies may inhibit the agglutinating effect of a serum on Rh-positive cells suspended in saline solution in at least two ways: either by its action on the cells or its action on the agglutinating serum.

The mode of action of the blocking antibody is of the greatest importance in interpreting the results of the albumin test. Is Diamond and Denton's[5] suggestion that the titer of blocking antibodies can be determined by calculating the difference between the saline and albumin titers correct? It seems reasonable to assume that the saline titer may not represent the total Rh agglutinin titer but may be a measure of the excess of effective Rh agglutinins while further agglutinins may be included in the antibodies estimated by the difference between the saline and albumin titers. This difference might, in fact, really be an estimate of "blocked agglutinins" and blocking antibodies together.

Before the validity of the assertions of various authors can be judged, it is necessary to consider the different possible hypotheses of the mode of action of the blocking antibody and the consequences expected from each. Then the results expected can be compared with those observed, and some assessment made of the consistency of each theory with the data. It should be possible to devise critical experiments which will show which, if either, postulated mode of action occurs in any particular case. The two modes of action considered, and their theoretically expected effects, will now be described.

1. *Adsorption on Cells.*—It has been observed that the addition of blocking antibody to Rh-positive cells will coat them and prevent their being subsequently clumped by agglutinins, either completely or partially. Two possible types of action may occur. First, the blocking antibody, even when added to the cells in mixture with the agglutinating antibody, may be preferentially adsorbed onto the cells, thus inhibiting clumping. Second, there could be competitive adsorption. This would result in complete coating of the cells if a sufficient concentration of the blocking antibody is added first, as in Wiener's original blocking test. However, only partial blocking will occur if the blocking and agglutinating antibodies are added in a mixture when there is competition for the sites on the cells on which they are to be adsorbed. This distinction could be obscured in practice if the agglutinins and blocking antibodies had different adsorption rates.

(a) *Preferential adsorption:* When the blocking antibody is preferentially adsorbed, it will inhibit clumping provided it is present in sufficient concentration, but may not completely inhibit clumping if it is diluted. Hence, a prozone effect should occur when successive dilutions of mixture are treated with the Rh-positive cells. This may be shown by the following argument.

Consider the effect of adding to unit quantity of cells a mixture containing A units of agglutinin and B units of blocking antibody, the units being so defined that one unit of agglutinin is sufficient to agglutinate unit amount of cells, and one unit of blocking antibody is sufficient to block unit amount of cells. Also, let *d* designate a dilution of the mixture such that unit volume of the original mixture is contained in a total volume d of final mixture; unit volume of the final mixture thus contains 1/d of the original. In titrating the mixture in successive dilutions against unit amount of cells, the blocking antibody will be adequate to cause complete blocking as long as d does not exceed B. For higher dilutions, the proportion of cells blocked will be only B/d, so that, provided the agglutinin is in sufficient concentration, the proportion of cells agglutinating will be 1–B/d. If the amount of agglutinin A is not sufficient at dilution d to agglutinate all unblocked cells, then only a proportion A/d will be agglutinated. The attached diagram (Fig. 1) sets out the expected

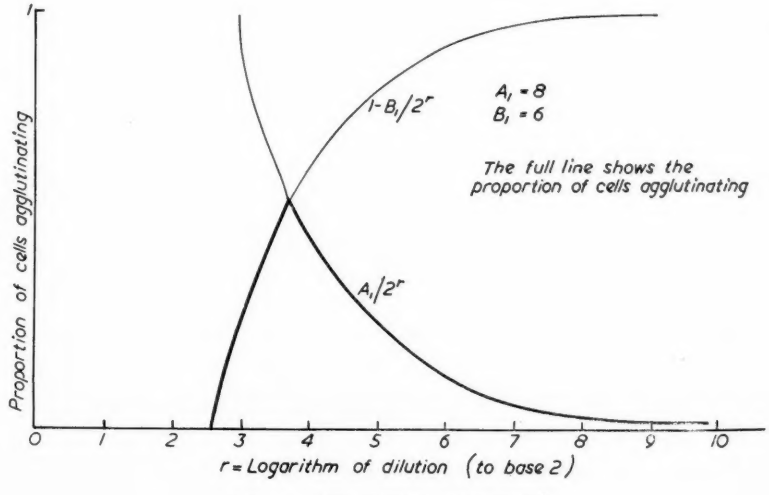

Fig. 1.—Preferential adsorption.

effect of dilution on the agglutinating effect; for the particular example, A = 8 and B = 6. From the diagram, two points may be noted: (1) the amount of agglutination rises to a maximum value when 1–B/d = A/d; then d = A + B and the proportion of cells agglutinated is A/(A + B); and (2) at high dilutions the agglutinating effect is dependent solely on A and is unaffected by the magnitude of B. It follows from the second point that the saline titer of a mixture, which is determined as the highest dilution for which there is any observable clumping, is simply a multiple of A, the multiplying

factor depending on the minimum concentration required. Thus, the presence of a blocking antibody which acts by preferential adsorption cannot be detected by its effect on an agglutinating serum in a saline titration. The amount of blocking antibody B required to mask completely the agglutinating effect must be sufficient to make the fraction $A/(A + B)$, the maximum agglutinating fraction, so small as to be undetectable. Theoretically, it should be possible to determine B from the prozone as the highest dilution for which no clumping occurs, but in practice, as explained below, this does not seem possible.

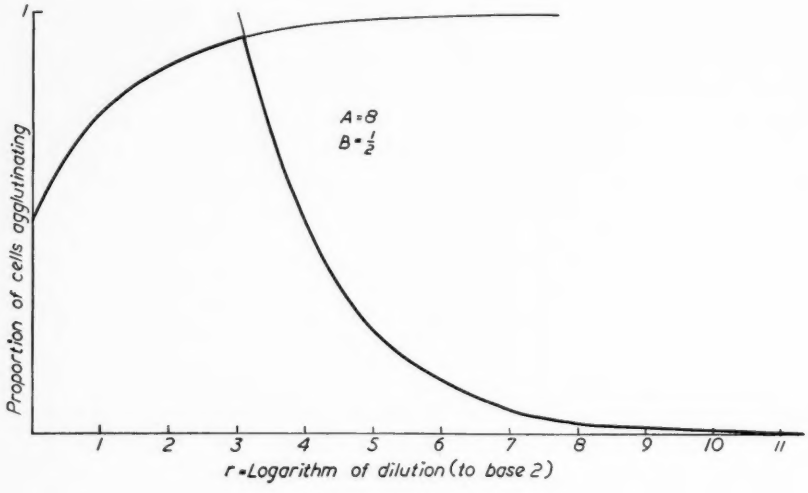

Fig. 2.—Preferential adsorption.

The mode of action of blocking antibodies by preferential adsorption is the one generally accepted. However, while it entails the occurrence of prozone as a necessary consequence, only rarely has the occurrence of prozone been reported in the literature. We have carefully examined 100 sera containing univalent antibodies and have failed to find one example of prozone. There are several reasons why this should be so. First, if the blocking antibody in a mixture is weak, $B < 1$ (as seems likely to be true usually; see Part II), there will be agglutination at all dilutions, and the prozone may pass unnoticed. This possibility is illustrated in Fig. 2, for which $A = 8$, $B = \frac{1}{2}$. Second, if the agglutinin is weak and the blocking antibody strong, the agglutinating effect may be masked at all dilutions. Thus, it is seen that while occurrence of prozone follows in all cases from the theory, the observation of prozone may be rare.

(b) *Competitive adsorption:* If the agglutinin and blocking antibody compete for sites on the cells to which they are to be adsorbed, the effect observed will be different, depending on whether the blocking antibody is added prior to the agglutinin or in mixture with it. With prior addition of blocking antibody, the effect will be exactly the same as with preferential adsorption, as described previously. Thus, the technique by which blocking serum is added

before the agglutinin cannot distinguish between preferential and competitive adsorption as modes of action of blocking antibody. Wiener's original blocking test is actually one of prior adsorption which may be competitive rather than preferential when a serum containing both blocking and agglutinating antibodies is added to the cells. Under these conditions there will be some agglutination at all dilutions in the range giving observable effects. The amount of agglutination given by the lower dilutions of the mixture will depend on the relative proportions of blocking and agglutinin in the mixture and on their relative avidity for sites on the cells. However, at high dilutions which are those from which the titer value is determined, the effect will be exactly the same as that described for preferential adsorption, since at high dilutions the competition between the antibodies will not be effective. Hence, for both modes of action by adsorption, the saline titer will be a measure of the amount of agglutinin present.

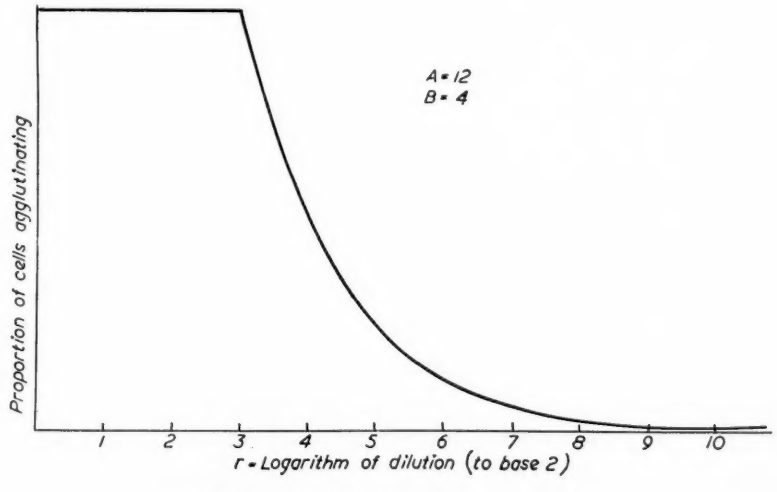

Fig. 3.—Neutralization of agglutinin.

2. *Neutralization of Agglutinin.*—An alternative mode of action which has not been considered by other workers is that blocking antibody may in some way neutralize the agglutinin, rendering it incapable of reacting with the cells. In this case, the blocking effect will be dependent on the relative amounts of blocking antibody and agglutinin in the mixture, and the curve of the agglutinin titer will be of the type shown in Fig. 3, which gives the example A = 12, B = 4, a unit of blocking antibody now being taken as the amount required to neutralize one unit of agglutinin. The results will be the same whether the blocking antibody is added before the agglutinin or in mixture with it. There will be no prozone effect with the mixture, since both reacting components will be diluted in the same proportion.

The blocking effect will not be revealed directly by the saline solution titer curve, since the curve will be identical with one for which the agglutinin

present is A–B. The presence of a significant amount of blocking antibody in a serum may be estimated by mixing it with an agglutinating serum of known titer and observing the decrease in titer in the mixture.

Conclusions.—From this discussion it appears that, on the adsorption hypothesis, the saline titer of a mixture represents the total Rh agglutinin titer; on the neutralization hypothesis, it represents the excess of effective Rh agglutinins.

Failure to realize the significance of these observations appears to be responsible for some confusion in the literature about the mode of action of blocking antibodies. Thus, some writers state that the action of the blocking antibodies is by adsorption on the cells, but they assume effects, such as an actual observable blocking of agglutinins persisting at high dilutions of the mixture, which are consonant only with the neutralization hypothesis.[5] On the other hand, blocking by adsorption probably occurs in some instances, as is shown by several reports in the literature of the observation of prozone.

The decision as to whether a blocking antibody acts by adsorption or neutralization is of vital importance in the interpretation of the results of the albumin titer. In any serum containing both saline agglutinins and blocking antibodies, it is difficult to demonstrate the presence of a blocking antibody acting by adsorption. This can be done only by observation of prozone which, as already stated, is of rare occurrence. The detection of a blocking antibody acting by neutralization is dealt with in Part IV.

It is felt in the light of this analysis that further critical examination of results reported in the literature may prove fruitful, but such a survey is beyond the scope of this paper.

PART II

INVESTIGATION OF THE MODE OF ACTION OF Rh ANTIBODIES

Since sera which proved to contain blocking antibodies by the original Wiener blocking technique[1] gave positive reactions in the albumin test, it has been assumed, following Diamond and Denton,[5] that all antibodies other than the saline agglutinins detected by this test must be blocking antibodies. We queried the correctness of this assumption after repeated failures to eliminate the effect of anti-D agglutinins by the addition of sera with high albumin titers which should have contained high concentrations of blocking antibodies. It seemed probable from these observations that the albumin test might also detect an incomplete antibody without blocking properties, and experiments were devised to examine this concept of two types of these antibodies, one with, and the other without, blocking properties.

In order to determine the mode of action of Rh antibodies in different tests, it is necessary to establish a basis against which observed results can be tested. It will be assumed, pending further evidence, that the agglutinin has the same clumping action in serum-albumin medium as it has in saline solution. The blocking antibody which will subsequently be referred to as Type 1 incomplete antibody has, in serum-albumin, no inhibiting effect on the agglutinin (either by adsorption on the cells or by neutralization) but itself acts

to clump the cells. It will be assumed that a unit of blocking antibody as defined in Part I has, in serum-albumin, a clumping effect equal to k times that of a unit of agglutinin. It will also be assumed that antibodies exist which neither block nor agglutinate in saline solution, but which cause agglutination in serum-albumin. These will be designated Type 2 incomplete antibodies. Thus, a mixture containing A units of agglutinin, B units of blocking, and C units of the third type of antibody will have, in serum-albumin, an expected titer of A + kB + C. These assumptions are adequate to account for a wide range of possible effects.

In order to test these possibilities and also to determine the relative potencies of the different components, a series of experiments was carried out, the experience gain in the preliminary ones aiding in the design of the subsequent ones.

A. *Experimental.*—

1. *Preliminary experiments:* We had at first assumed, following Diamond and Denton,[5] that a serum with a high serum-albumin titer but a very low saline titer was a blocking serum. However, only once have we been able to eliminate the effect of an anti-Rh_0 (anti-D) antibody from an anti-Rh_0' (anti-CD) serum by using such a presumed blocking serum. It was necessary to add an equal volume of the blocking serum to produce this effect, and further investigation showed that similar dilution with saline solution or serum of low conglutinin titer was equally satisfactory. It was obvious that the Rh_0 agglutinin was present in low concentration and, since the effect of adding blocking antibody was no different from that of adding saline solution, it would appear to have been a mere dilution effect rather than one of adsorption or neutralization.

Two other sera containing only anti-Rh_0 (anti-D) antibodies, of which the titer was 16, were mixed, in proportions varying from 1 in 10 to 1 in 2, with three sera having serum-albumin titers of 32, 32, and 64, and, subsequently, with a number of other sera presumed to be potent blocking sera. In every case, agglutination occurred with intensity comparable with that obtained by simple dilution of the anti-Rh_0 (anti-D) serum with saline solution. If these sera had any blocking effect it certainly was not one of neutralization since the titer of the anti-Rh_0 (anti-D) sera was not reduced. These sera either contained no blocking antibody or had only a low concentration of blocking antibody which acted by adsorption. If this last possibility were true, the cells would only be partially blocked so that some agglutination could occur and the saline titer would not be affected.

Besides failing to eliminate the effect of saline agglutinins with some sera containing presumed blocking antibodies, we did not consider that the data presented in Diamond and Denton's paper[5] were reported in sufficient detail to establish clearly that the concentration of blocking antibodies could be assessed validly from the difference of albumin and saline titers. We, therefore, decided to conduct experiments to determine the nature and mode of

TABLE I. COMPARISON OF THE OBSERVED AND CALCULATED SALINE AND SERUM-ALBUMIN TITERS OF A SERUM CONTAINING RH AGGLUTINATING ANTIBODIES AND ANOTHER PRESUMED TO CONTAIN BLOCKING ANTIBODIES, BOTH ALONE AND IN MIXTURES

COMPOSITION OF TEST SERA IN UNITS OF 1/3 ORIGINAL CONCENTRATION		EXPECTED RESULT		EXPERIMENT 1				EXPERIMENT 2			
				SALINE TITER		SERUM-ALBUMIN TITER		SALINE TITER		SERUM-ALBUMIN TITER	
"AGGLUTINATING" SERUM	"BLOCKING" SERUM	SALINE	SERUM-ALBUMIN	OBS.	CALC.	OBS.	CALC.	OBS.	CALC.	OBS.	CALC.
3	0	$3A_1$	$3(A_1 + kB_1)$	57	59.42	320	252.45	91	84.18	512	584.82
2	0	$2A_1$	$2(A_1 + kB_1)$	40	39.62	160	168.30	45	56.12	362	389.88
1	0	A_1	$(A_1 + kB_1)$	20	19.81	80	84.15	16	28.06	256	194.94
0	3	–	$3kB_2$	–	–	80	105.85	–	–	128	120.81
0	2	–	$2kB_2$	–	–	80	70.56	–	–	64	80.54
0	1	–	kB_2	–	–	40	35.28	–	–	45	40.27
2	1	$2A_1$	$2(A_1 + kB_1) + kB_2$	40	39.62	160	203.58	64	56.12	128	430.15
1	2	A_1	$(A_1 + kB_1) + 2kB_2$	20	19.81	113	154.71	32	28.06	256	275.48

The titer value given is the dilution for which the response is a very weak agglutination. Where two dilutions give this response, or where the response is a stronger agglutination followed by a negative reaction, the geometric mean of these two dilutions is used, since the dilutions are in geometric progression.

Obs. = observed; calc. = calculated.

action of these incomplete antibodies. The method adopted was to test the effects of mixtures containing varying proportions of agglutinating and blocking antibodies.

2. *Experiments to determine the mode of action of Rh antibodies:*

(a) *Initial experiments using mixtures of sera containing agglutinins with sera containing incomplete antibodies.* In these experiments, titrations with cells in saline solution were carried out on an agglutinating serum and a serum containing incomplete antibodies with little, if any, saline agglutinins, each at three different concentrations and on mixtures of the two sera. For each titration of each serum or mixture, dilutions of 1 in 5, 1 in 10, and so on, in geometric progression, were used. In later experiments, dilutions of 1 in 2, 1 in 4, and so on, were used.

For the serum-albumin titer, the serum under investigation was diluted with serum, and a 4:1 serum-albumin mixture was used as suspension medium for the cells. We have found that pooled serum incubated at 37.5° C. overnight, especially when fortified with albumin in the proportion of 4:1, is a more satisfactory suspension medium than plasma, with which there is often intense rouleaux formation. Even better results are obtained by suspending the cells in human or bovine albumin.[6] Until recently, however, albumin supplies have been limited so that it has been necessary to use serum-albumin mixture for the conglutination titers reported in this section.

Two experiments of this type were carried out using different sera. The compositions of the test sera were as set out in the first two columns of Table I, which also gives the results of the saline and serum-albumin titrations. These results have been statistically analyzed in the first instance on the assumption that they are consistent with the adsorption hypothesis and that the agglutinating serum at 1 in 3 concentration contains A_1 units of agglutinin and B_1 units of blocking antibody while the incomplete serum at 1 in 3 concentration contains B_2 units of blocking antibody. The possibility that antibodies are present which neither block nor agglutinate cells in saline solution, but which agglutinate in serum-albumin (that is, Type 2) has been ignored. For the analysis it has been assumed that the standard deviation of a result is proportional to the value expected.

TABLE II. ESTIMATES OF CONSTANTS AND THEIR STANDARD ERRORS ON THE ASSUMPTION THAT BLOCKING IS BY ADSORPTION

VALUES CALCULATED FOR	EXPERIMENT 1	EXPERIMENT 2
A_1	19.8 ± 2.2	28.1 ± 3.1
kB_1	64.3 ± 10.8	166.9 ± 23.6
kB_2	35.3 ± 4.9	40.3 ± 5.7

The results expected on these assumptions are tabulated in Table I, along with the results observed. They are calculated from the estimates of A_1, kB_1 and kB_2 set out in Table II. The standard deviation of individual results from those expected is 25 per cent, which is not excessive for data of this type. The results show excellent agreement between observation and calculation.

The observed results can be interpreted in two ways. They are compatible with the presence of a blocking antibody acting by adsorption, but it is curious that, if this hypothesis holds, no prozone has been observed. The results can be assumed equally well to indicate that no significant amounts of blocking antibody are present, but that the so-called "blocking sera" contain a non-blocking type of antibody revealed by the serum-albumin titrations. Second, the data were analyzed on the assumption that the blocking antibody had a neutralizing effect on the agglutinin; in this analysis it was found that the potency of the blocking antibody in saline solution was nil. This result means that no incomplete antibodies acting by neutralization were present in these sera.

(b) *Further experiments using mixtures of sera containing agglutinins with sera containing incomplete antibodies which in some instances were proved to include blocking antibodies.* Because other workers had repeatedly observed that mixture of agglutinating and blocking sera resulted in a marked reduction in the agglutinating titer (which on the theory presented in Part I could only be by neutralization), it was decided to test certain presumed blocking sera for the existence of this effect. The 36 sera listed in Table III, most of which had been stored in a refrigerator for many months, were tested by the original Wiener blocking test as well as by saline and serum-albumin agglutination tests, with the object of deciding which sera definitely contained significant concentrations of blocking antibody for agglutinins.

In order to detect the presence of blocking antibodies in these sera by the original Wiener technique and also to render them usable as blocking sera, it was necessary first to destroy agglutinating antibodies in many of them. This was accomplished by heating.

Diamond and Abelson[3] stated that heating sera containing Rh agglutinating antibodies for several hours at 56° C. will result in their complete destruction. Wiener and co-workers[7] found similar results could be produced by heating at 60° C. for 30 minutes, and Haberman, Reid, and Hill[8] found heating at 70° C. for 10 minutes satisfactory.

In this laboratory, heating at 70° C. was found to convert many sera into jelly useless for further tests, while heating even for one hour at 60° C. failed to destroy agglutinating antibodies completely. For this reason, the effect of temperature on both the incomplete antibodies and saline agglutinins in a particular serum was investigated. Saline and serum-albumin titers were performed on serial dilutions of four portions of Serum J (1) heated for 18 hours at 56° C., (2) heated for 30 minutes at 60° C., (3) heated for 10 minutes at 70° C., and (4) unheated. The results may be summarized as follows:

1. Heating for 18 hours at 56° C. often completely destroyed the agglutinating antibodies; there was no evidence of any destruction of the incomplete antibodies.
2. Heating for 30 minutes at 60° C. did not completely destroy agglutinating antibodies; there was no significant effect on the incomplete antibodies.
3. Heating for 10 minutes at 70° C. completely destroyed agglutinating and partially affected the incomplete antibodies.

It appears, then, that agglutinating antibodies are more susceptible to high temperatures or long heating at lower temperatures than are incomplete ones.

In several experiments on other sera it was found that heating at 60° C. for one hour was insufficient to destroy the saline agglutinins. In some cases in which the agglutinin titer was high, heating at 56° C. for as long as 72 hours was necessary to destroy them completely. Similar observations have been made by Jakobowicz.[9]

The results recorded in Table III show that blocking antibody was detected by the original Wiener technique in only 14 of the 36 sera tested. In no case was the titer greater than 12, yet in one instance (No. 24) the serum-albumin titer was greater than 500. In other cases, for example, Nos. 2, 7, 9, and 15, in which no blocking antibody was detected by the original Wiener technique, the serum-albumin titer exceeded 1,000. These results could be explained by the suggestion of Wiener and Gordon[10] and Wiener and Katz[11] that the original method is less sensitive than the serum-albumin method. They found that a titer of 40 by the serum-albumin technique is necessary to obtain blocking by the original method. However, our suggestion that there exists a nonblocking, as well as a blocking, incomplete antibody offers an alternative explanation and makes it doubtful whether a difference in sensitivity of the two methods is sufficient to explain the difference in titer of the "blocking" antibodies.

TABLE III. COMPARISON OF THE TITERS OF INCOMPLETE ANTIBODIES ASSESSED BY THE SERUM ALBUMIN AND THE ORIGINAL WIENER BLOCKING TECHNIQUES

	TITERS		
SERUM NO.	SALINE SOLUTION	SERUM-ALBUMIN	BLOCKING—ORIGINAL WIENER TECHNIQUE
1	2	16	0
2	1	2,000	0
3	1	32	0
4	1	16	0
5	2	256	Weak
6	4	512	0
7	1	2,000 +	1-2
8	0	8	0
9	8	2,000	0
10	0	32	? Weak
11	0	256	0
12	2	8	0
13	0	512	0
14	8	16	0
15	0	2,000	0
16	0	16	0
17	0	512	0
18	0	16	0
19	32	1,000	0
20	1	64	8
21	1	16	0
22	0	128	3
23	0	256	12
24	0	1,000	6
25	0	16	8-16
26	0	32	4
27	1	32	Weak
28	0	128	12
29	32	512	? Weak
30	32	256	12
31	2	128	12
32	1	64	5
33	2	128	6
34	0	4	0
35	0	1	0
36	16	64	0

To obtain evidence as to whether this idea of two types of incomplete antibodies is tenable, a further series of tests was performed and submitted

TABLE IV. COMPARISON OF SALINE, SERUM, AND SERUM-ALBUMIN TITERS OF A SERUM CONTAINING RH AGGLUTINATING ANTIBODIES AND OTHERS CONTAINING INCOMPLETE ANTIBODIES WHICH INCLUDED THE BLOCKING TYPE WHEN TESTED BY THE ORIGINAL WIENER BLOCKING TECHNIQUE, BOTH ALONE AND IN MIXTURES

EXPERIMENT		1			2			3			4		
COMPOSITION OF TEST SERA		BLOCKING SERUM NO. 20			BLOCKING SERUM NO. 23			BLOCKING SERUM NO. 25			BLOCKING SERUM NO. 28		
AGGLUTINATING NO. 29	BLOCKING	SALINE	SERUM	SERUM-ALBUMIN	SALINE	SERUM	SERUM-ALBUMIN	SALINE	SERUM	SERUM-ALBUMIN	SALINE	SERUM	SERUM-ALBUMIN
1	0	32	32	512	23	45	256	23	11	362	6	0	32
2/3	0	8	16	128	11	16	45	23	8	91	8	0	64
1/3	0	8	8	64	8	16	45	8	2	32	6	0	64
0	1	0	16	256	0	16	45	0	2	128	0	0	128
0	2/3	0	8	64	0	1	181	0	2	128	0	0	64
0	1/3	0	4	64	0	4	45	0	0	23	0	0	16
2/3	1/3	11	64	256	2	4	128	6	23	256	4	0	64
1/3	2/3	2	45	512	1	8	128	4	4	256	1.4	0	64
Titer by Wiener's original blocking test			8			12			10			12	

The titer value given is the dilution for which the response is a very weak agglutination. Where two dilutions give this response, or where the response is a stronger agglutination followed by a negative reaction, the geometric mean of these two dilutions is used, since the dilutions are in geometric progression.

TABLE V. COMPARISON OF SALINE, SERUM, AND SERUM-ALBUMIN TITERS* OF A SERUM CONTAINING RH AGGLUTINATING ANTIBODIES AND OTHERS CONTAINING INCOMPLETE ANTIBODIES WHICH DID NOT INCLUDE THE BLOCKING TYPE WHEN TESTED BY THE ORIGINAL WIENER BLOCKING TECHNIQUE, BOTH ALONE AND IN MIXTURES

COMPOSITION OF TEST SERA		EXPERIMENT 1 BLOCKING SERUM NO. 13			EXPERIMENT 2 BLOCKING SERUM NO. 16			EXPERIMENT 3 BLOCKING SERUM NO. 36		
AGGLUTINATING NO. 29	BLOCKING	SALINE	SERUM	SERUM-ALBUMIN	SALINE	SERUM	SERUM-ALBUMIN	SALINE	SERUM	SERUM-ALBUMIN
1	0	16	128	362	16	8	45	23	16	128
2/3	0	16	32	256	22	11	64	32	8	64
1/3	0	6	11	128	8	2	16	11	3	32
0	1	0	4	256	0	2	16	0	1.4	8
0	2/3	0	4	512	0	2	16	0	0	6
0	1/3	0	8	256	0	1.4	11	0	0	4
2/3	1/3	16	32	256	16	8	91	32	16	256
1/3	2/3	6	16	2,048	8	6	45	11	4	64

Original Wiener Blocking test was negative in all three sera.
*See footnote, Table IV.

to statistical examination. Out of the group in Table III, seven sera were selected: Nos. 20, 23, 25, and 28, because they contained blocking antibody when tested by the original Wiener prior-adsorption method and had serum-albumin titers varying from 16 to 256; No. 13 because the serum-albumin titer was 512, yet the original Wiener technique failed to detect blocking antibody; and Nos. 16 and 36 because they gave no positive reaction with the original Wiener technique and had only moderate serum-albumin titers.

Experiments similar to those already described in section *1* (*a*) were carried out on each of these seven sera. One agglutinating serum (No. 29) which also contained incomplete antibodies was used in combination with each of the sera being tested for blocking antibodies. The results are recorded in Tables IV and V.

It will be noticed that the titers for this serum in the different media vary slightly from assay to assay. These variations are due to the fact that the same ampule of serum No. 29 could not be used for each of the seven assays and individual ampules which had been stored for over twelve months showed slight differences in titer. Such variations are not uncommon in other branches of serologic work, for example, decrease of titer in hemolysin for Wassermann tests. Wiener suggests that the variations in titers in different ampules could be due to the high order of inaccuracy inherent in these titrations.

B. *Method of Analysis.*—Before undertaking an interpretation of the results, the method of analysis will be set out in some detail.

If the theoretically expected titers are designated as follows:

	No. 29	*Other Sera*
Saline	3 U	3 X
Serum	3 V	3 Y
Serum-albumin	3 W	3 Z

then the mixtures provide estimates of:

2 U + X	U + 2 X
2 V + Y	V + 2 Y
2 W + Z	W + 2 Z

From these estimates, values for each of the six unknowns can be derived. Any neutralizing effects will be revealed in discrepancies between these estimates and those for unmixed sera (Table VI). The results of these experiments, recorded in Tables IV and V, have not been analyzed in such detail as those given in Table I. It was realized that a full analysis, as given in Tables I and II, was not necessary for establishing the points we wished to make; the analysis given there does, however, show the possibilities of the method.

Also, a method of analysis more satisfactory than that used for examining the results in Table I has been adopted. Instead of weighting the titer values inversely as the squares of their expectations (the variance of an observation having been assumed proportional to the square of its expectation), we have converted the observed values to logarithms, which may be expected to have approximately constant variability. Since some of the values are zero, we have actually used $\log_{10}$ (titer value + 1), but, in practice, this adjustment is negligible. In the following discussion, and in Table VI, the estimates of logarithmically transformed titers will be denoted by lower-case letters.

The standard errors of the logarithmic estimates from the individual and mixed sera are found as follows. The results for the unmixed sera provide an

estimate, denoted by S^2, of the variance of a transformed observation. The variance of any logarithmic estimate from unmixed sera, being based on three observations, is given by $S^2/3$. For the mixed sera, the formulas are more complicated. Since X is found to be consistently zero, the estimate w, from the two mixtures tested in saline solution, is $S^2/2$. The same variance applied to the estimates of v, whenever Y can be assumed zero. When neither component of a mixture is zero, the variance of the logarithmic estimate is given by formulas of the following type:

$$\text{Var (Wm)} = \frac{S^2}{9} \frac{[\,4\,(2W+Z)^2+(W+2Z)^2\,]}{W^2}$$

$$\text{Var (Zm)} = \frac{S^2}{9} \frac{[\,(2W+Z)^2+4(W+2Z)^2\,]}{Z^2}$$

From these formulas, the standard errors of differences between any two estimates can be determined and the difference can then be tested for significance. The conclusions drawn from the significant differences found are discussed below.

C. *Interpretation of Results.*—

(a) *Presence of Type 1 incomplete antibody (blocking for agglutinins) acting by neutralization.* Since no prozone has been observed in the experiments, they give no evidence for the presence of antibodies blocking by preferential adsorption. Any blocking antibodies present in observable quantities must act by neutralization. Such a neutralizing effect would be discovered by comparing the saline titer (u) estimated from the mixtures (Um) with that determined from the agglutinating serum No. 29. From inspection of Table V, there is no neutralizing effect apparent in the mixture of the agglutinating serum No. 29 with sera Nos. 13, 16, or 36. It is significant that no blocking antibody was detected in any of these sera by the original Wiener prior-adsorption technique, although according to the serum-albumin technique, incomplete antibodies of titers 256, 64, and 16 were present in the respective sera.

From Table IV, sera Nos. 20, 23, 25, and 28, with incomplete antibody titers of 256, 64, 128, and 128, respectively, appeared to show some neutralizing action, but statistical analysis revealed that a significant effect is found in the experiment on serum No. 23 only. This effect for serum No. 23 is significant even after allowance is made for the fact that it is the largest of seven observed differences. It thus appears to be a real neutralizing action, but since it has been observed only once in these experiments, it cannot be regarded as of common occurrence. It is interesting to note that serum No. 23 had an albumin titer of only 64 while Nos. 13, 20, 25, and 28 had titers of 256 and 128.

(b) *Presence of Type 2 incomplete antibody.* The results presented in Tables IV and V show that there is a marked difference between the serum-albumin and saline titers, yet we have failed to demonstrate the presence of a significant amount of blocking antibody in any except serum No. 23. We therefore conclude that a nonblocking type of incomplete antibody which we designate Type 2 is present in these sera.

(c) *Presence of neutralizing antibody Type 3 for Type 2 incomplete antibodies.* The difference in titers with serum and serum-albumin as presented in Table VI was next examined. In these tests, the values in serum-albumin

TABLE VI. TABLE OF LOGARITHMIC ESTIMATES

SERA	SOURCE OF ESTIMATE	u	v	w	y	z
No. 29	Unmixed	0.86	1.32	2.10	0.60	2.25
No. 13	Mixed	0.89	1.23	0.00	0.00	2.71
	SE of difference	0.18	0.31	0.11†	1.32	0.18
No. 29	Unmixed	0.95	0.62	1.32	0.30	0.95
No. 16	Mixed	0.95	0.65	1.66	0.32	0.00
	SE of difference	0.18	0.36	0.34	0.63	0.82
No. 29	Unmixed	0.91	0.99	1.95	0.73	1.76
No. 20	Mixed	0.65	1.46	0.00	0.99	2.41
	SE of difference	0.18	0.37	0.47†	0.60	0.42
No. 29	Unmixed	0.90	1.23	1.66	0.56	1.61
No. 23	Mixed	0.30	0.00	1.64	0.70	1.64
	SE of difference	0.18†	0.34†	0.43	0.75	0.46
No. 29	Unmixed	0.99	0.62	1.76	0.18	1.61
No. 25	Mixed	0.64	0.89	1.94	0.00	1.94
	SE of difference	0.18	0.18	0.40	0.11	0.51
No. 29	Unmixed	0.73	-	1.47	-	1.46
No. 28	Mixed	0.43	-	1.35	-	1.35
	SE of difference	0.18	-	0.44	-	0.45
No. 29	Unmixed	1.08	0.69	1.56	0.06	0.62
No. 36	Mixed	1.16	0.83	1.96	0.00	0.00
	SE of difference	0.18	0.18	0.18*	0.11	0.11†

*Difference significant at 5 per cent level.
†Difference significant at 1 per cent level.

exceed those in serum in every case. The standard error of the difference ($W_p - V_p$ for No. 29 or $Z_p - Y_p$ for the others) is 0.157, and the difference required for significance at the 1 per cent level is 0.42. The differences for each experiment all exceed 0.42 and are therefore significant. Since the antibody blocking by neutralization has been shown to be absent from all sera except No. 23 and antibody acting by preferential adsorption has been shown to be absent or weak by the absence of prozone, these results can be interpreted only as again confirming the accepted opinion of most workers that serum-albumin is a more suitable medium for the detection of incomplete antibodies than is serum alone.

The titers derived from results for unmixed serum No. 29 in serum-albumin differ from those from mixtures with other sera only for Nos. 13 and 20. The effect in these cases appears to be that serum No. 29 is completely inhibited when admixed with Nos. 13 and 20.

When tested with Rh-positive cells in saline solution, neither of these sera gave any indication of containing agglutinating antibodies or blocking antibodies for agglutinins. However, it appears from these results that they contain an incomplete antibody which we shall designate Type 3, which can inhibit the effect of both agglutinins and Type 2 incomplete antibodies when tested with Rh-positive cells in albumin. This antibody is not a blocking antibody in the sense usually understood and appears to have a neutralizing effect.

The findings presented in this section are discussed in detail in Part III.

PART III

FIVE FORMS OF Rh ANTIBODIES

The experimental work described in Part II proves that the original division of Rh antibodies into the saline agglutinins and blocking antibodies is no longer sufficient to explain recent observations.

In Part II we demonstrated, by investigation of mixtures of a serum containing saline agglutinins with others containing incomplete antibodies, that these antibodies were divisible into at least three types. As well as the blocking antibody for saline agglutinins (Type 1), there is an antibody without blocking properties (Type 2), and also Type 3, a blocking antibody for Type 2. We have evidence also that there is still another type of antibody which reacts with the Coombs' reagent but is not detected by the albumin method.

The saline agglutinins need no further comment. The evidence for the existence of the other four types will now be examined in relation to the work of other investigators.

A. *Evidence for Four Types of Rh Antibody in Addition to Saline Agglutinins.—*

1. *Type 1 incomplete antibodies–blocking antibodies:* The generally accepted theory regarding the mode of action of blocking antibodies is that of preferential adsorption which was first suggested by Wiener. As has been shown in Part I, sera containing saline agglutinins and significant amounts of blocking antibody acting by preferential adsorption should exhibit prozone. Various workers[1, 2, 13, 14, 15, 16] have observed prozone in individual sera, but, in practice, prozone occurs infrequently. We have failed to find a single serum exhibiting the prozone phenomenon in more than 100 sera examined.

In Part I we described another theory, namely, that the blocking antibody may act by neutralization of the agglutinins. From the evidence presented by other workers and in Parts I and II of this paper, it seems certain that incomplete antibodies having the power to inhibit the action of saline agglutinins do exist and that some of them act by preferential adsorption and others by neutralization. However, blocking antibodies exerting their influence in either of these ways occur infrequently in significant amounts, even in sera with high albumin titer.

2. *Type 2 incomplete antibodies:* The assumption that all incomplete antibodies estimated in conglutination tests were blocking antibodies has arisen because of the order of discovery of the original Wiener blocking test and the conglutination test. Since sera which contained blocking antibodies when they were tested by Wiener's original prior adsorption technique were found to give positive conglutination reactions, it was assumed that the converse was also true, namely, that sera giving positive conglutination reactions must contain blocking antibodies.

In Part II we showed that the effect of saline agglutinins was not eliminated by the addition of a number of different sera, some with low and others with high albumin titers. These experiments lead to the conclusion that either blocking antibodies must be present in very high concentration in order to inhibit

saline agglutinins or that the albumin test can also detect other antibodies without blocking properties. Simmons[17] found that equal volumes of a blocking serum would not eliminate the effects of agglutinating sera of comparable albumin titer. In his experience, one, two, or three volumes of "blocking" serum of extremely high albumin titer are needed to eliminate the effect of one volume of agglutinating serum. He suggested that the sera used in our experiments were too weak. However, our serum No. 23, with a conglutination titer of only 64, proved to be effective because it was, in our interpretation, one in which the incomplete antibody was mainly of a true blocking type, acting in this case by neutralization. For the same reason, in the example quoted by Wiener, Davidson, and Potter[18] two parts of anti-Rh_0 (anti-D) blocking serum of titer 64 appears to have been sufficient to convert their anti-Rh_0' (anti-CD) serum into an anti-Rh′ (anti-C) reagent. We feel, therefore, that when sera of very high serum-albumin titer are necessary to exert a blocking effect, it may well be that there is a mixture of some blocking antibody with a greater proportion of a different incomplete antibody without blocking properties.

Another line of argument for the existence of the second type of incomplete antibody is derived from consideration of the sera used in Rh testing. In most instances, there is a usable titer of Rh agglutinins, but there is also a much higher serum-albumin titer. In one serum, at present being used in this laboratory, the total serum-albumin antibody titer is 32 times the saline agglutinin titer. If these incomplete antibodies were a blocking type acting by preferential adsorption, one would expect Rh-positive cells to be completely coated so that they would appear to be Rh-negative. However, this serum causes definite agglutination of the cells; it therefore seems reasonable to infer that a very high concentration of incomplete antibody with no blocking properties must be present. This antibody can be detected, and its concentration determined, by the serum-albumin technique.

A third argument in favor of the existence of more than one type of Rh incomplete antibody is the rarity of complete blocking of the child's red blood cells in spite of a high maternal serum-albumin titer. In only 19 out of 456 cases in which the maternal serum contained incomplete antibodies did the red blood cells of the child appear to be Rh-negative, while they were really Rh-positive, as shown by agglutination when the cells were suspended in serum-albumin. This blocking effect is quite independent of the level of the maternal serum-albumin titer since this ranged from 24 to over 500. On the other hand, even sera with very high titers do not necessarily produce a blocking effect. Our series contains four cases in which the maternal antibody titer was 1,000 to 2,000, yet the infants' cells gave the correct Rh-positive reaction.

Our experiments therefore make it quite clear that a serum may contain a nonblocking incomplete antibody which is detected by the serum-albumin technique. It exerts no blocking effect either by adsorption or by neutralization of agglutinins. The presence of this nonblocking Type 2 incomplete antibody instead of blocking antibody (Type 1) in so many sera explains our failure in early experiments to eliminate the effect of Rh saline agglutinins in sera containing these antibodies, alone or mixed with others, by the addition of a serum

with a high serum-albumin titer. Blocking antibodies appear to be present in significant amounts in only a proportion of the sera from mothers of erythroblastotic children, while this other type of incomplete antibody occurs in nearly all of them, often in high titer.

Hill, Haberman, and associates[19-21] reported a new type of antibody, the cryptagglutinoid. This differs from Rh saline agglutinins and also from blocking antibodies since it is capable of specific adsorption without causing either agglutination or blocking. Although not demonstrable by either the saline agglutinating or blocking tests, it can be detected by both the albumin and the indirect Coombs' methods. It is possible that our Type 2 incomplete antibody is identical with the cryptagglutinoid.

3. *Type 3 incomplete antibody–blocking for Type 2 and saline agglutinins:* As mentioned in Part II, sera Nos. 13 and 20 contained a second type of blocking antibody which was not capable of inhibiting saline agglutinins in serum No. 29 when tested with Rh-positive cells suspended in saline solution, but which eliminated the effect of agglutinins and Type 2 incomplete antibodies when the mixture of sera was tested with Rh-positive cells suspended in serum-albumin. Witebsky and Mohn[12] have reported a new blocking antibody which they isolated in the "supernatant" fraction of a serum which had been dialyzed for three successive 18-hour periods. This antibody produced an effect similar to that which we have observed. While no explanation for the mode of action of this third type of incomplete antibody is offered, it appears to be distinct from other types observed.

4. *Fifth type of Rh antibody:* Hill, Haberman, and associates[19-21] have reported that some sera which they thought contained cryptagglutinoids reacted in the Coombs' test only. Sturgeon[22] referred to this antibody as a cryptagglutinoid variant. We have also found in the sera of a small group of mothers whose babies developed erythroblastosis an antibody which is detectable by the Coombs' reaction after adsorption onto Rh-positive cells. This antibody fails to react when tested by the serum-albumin technique.

TABLE VII. COMPARISON OF RH ANTIBODY TITERS BY INDIRECT COOMBS' AND SERUM-ALBUMIN METHODS

INDIRECT COOMBS' TITER	NUMBER OF TESTS WITH SERUM-ALBUMIN TITERS													TOTALS
	0	1	2	4	8	16	32	64	128	256	512	1,024	2,048	
0	–	1	–	–	3	2	–	1	2	1	2	–	–	12
1	1	–	–	–	–	–	1	1	–	1	–	–	–	4
2	–	2	3	1	2	1	3	–	2	–	–	–	–	14
4	–	1	–	–	4	6	2	2	3	–	–	–	–	18
8	1	–	1	1	3	5	4	7	2	6	1	1	–	32
16	–	1	–	1	1	6	4	4	1	2	1	3	1	25
32	–	3	1	–	–	–	2	7	4	6	7	2	3	35
64	–	–	–	1	–	1	1	2	6	10	3	5	2	31
128	–	1	–	1	–	–	1	1	1	2	2	–	2	11
256	–	1	–	–	–	–	–	1	–	–	2	1	1	6
512	–	–	–	–	–	–	–	2	–	1	1	–	1	5
1,024	–	–	–	–	–	–	–	–	–	–	–	–	1	1
2,048	–	–	–	–	–	–	–	–	–	1	–	–	–	1
Totals	2	10	5	5	13	21	18	28	21	30	19	12	11	195

Hill and Haberman's report of an antibody which on some occasions reacted in both albumin and antiglobulin tests and on other occasions only with the latter, has always suggested to us the possibility that those sera giving both tests did not contain cryptagglutinoids only.

Analysis of serum-albumin and indirect Coombs' titers carried out on 195 sera from mothers of erythroblastotic babies gave evidence that this idea may be correct. We had undertaken this series of tests, the results of which are reported in Table VII, to clear up doubts regarding Wiener's claim that the Coombs' test may show, on the average, two to four times the sensitivity of the plasma-albumin method. Examination of Table VII shows that when using antiglobulin sera* the serum-albumin titer is generally higher than the indirect Coombs' titer, but that the relationship between the two is not close. The correlation between their logarithms is 0.42, which, while significant, is too low to be accepted as evidence that the titers are equivalent. Hence, it would appear that, as might have been expected, the two titers measure different properties of the serum and that a discussion of their relative sensitivities is therefore irrelevant.

This fifth type of antibody appears to be identical with a type revealed in Mohn and Witebsky's dialysis experiments[12, 24, 25] in which their third globulin fraction contained a low titer of incomplete (albumin) Rh antibodies, but had no blocking properties; nevertheless, Rh-positive cells sensitized with it gave a strong Coombs' reaction. The antibody reacting in this way was called by them Type 4. They suggested that washing had removed the incomplete Rh antibody so that the washed Rh-positive cells failed to show microscopically visible agglutination when suspended in undiluted normal adult human serum. The Coombs' titer with these washed cells was approximately 700 times the albumin titer in the globulin fraction No. 3.

The evidence strongly suggests the possibility that the indirect Coombs' test isolates this fifth type of Rh antibody, namely, the fourth type of Witebsky and Mohn, or the cryptagglutinoid variant of Hill and Haberman.

B. *The Significance of the Variability in Titers of Rh Antibodies by Different Methods of Estimation.*—With the increase in the number of methods for estimation of Rh antibodies it soon became obvious that the titers obtained by various methods in the same serum differed greatly. For example, Wiener's blocking test[1] is stated by most workers to give lower readings than any of the other methods. Henry and Simmons[6] found great variation in titers of individual sera. They found that Wiener's blocking test always gave the lowest titer and their own technique (serum-albumin) the highest. Wiener and Gordon[10] confirmed these findings. They also thought that the titers by the indirect Coombs' test and the plasma conglutination methods were of a similar order. Later Wiener[23] came to the conclusion that the indirect Coombs' test may give readings two to four times higher than the serum-albumin test. According to Hill, Haberman, and associates,[19, 20] the indirect Coombs' test gives a higher titer than the Wiener blocking and albumin tests.

*Prepared by Commonwealth Serum Laboratories, Australia.

These observations have been generally interpreted as indicating differences in sensitivity of the several tests. Proof for this assumption has never been offered, but there seemed to be no alternative explanation in the literature. However, the discoveries detailed previously, of at least five different forms of Rh antibody, suggested such an alternative. Would it not be better to explain the observations in terms of reactivity than of sensitivity? Instead of assuming that one test is more sensitive than the other, is it not more likely that one test gives a reaction with only one type of antibody, while another estimates a different type of antibody or even several of them?

The idea that a particular test is capable of estimating the titer of one component in a mixture of Rh antibodies in an anti-Rh serum has already been accepted in the case of saline agglutinins. Thus, although serum, plasma, or albumin conglutination techniques will detect both saline agglutinins and incomplete antibodies, only the saline agglutinins are estimated by testing the serum with saline-suspended Rh-positive cells. In view of the fact that it has been shown that incomplete antibodies may be of three kinds, Type 1 (blocking antibodies for agglutinins), Type 2 (no blocking properties), and Type 3 (antibodies blocking in serum-albumin), it seems logical to revise the idea that Wiener's blocking technique is less sensitive than the serum-albumin technique. It is possible that the former estimates the concentration of Type 1 antibodies only, while the latter estimates Type 2 if Type 3 is absent, but only Type 3 if both Type 2 and Type 3 are present as well as Type 1.

There seems to be a similar relationship between the serum-albumin technique and the indirect Coombs' test. Comparison of the Rh antibody titers by these two methods originally suggested to us that some antibodies were detected with equal facility by either method, others reacted more readily with the serum-albumin test and still others were revealed only by reaction with the Coombs' antihuman-globulin serum. The fifth type of antibody in the sera of some immunized mothers of affected children reacted strongly in the Coombs' test, yet only traces of or no incomplete antibodies could be detected by the serum-albumin method. In one instance, the titer by the indirect Coombs' method was 256 while there was only a very weak reaction with the undiluted serum in the serum-albumin test. On the other hand, we found some instances in which a high dilution of the mother's serum will still clump Rh-positive cells suspended in albumin, and yet the titer of antibodies by the indirect Coombs' method has been negative or very low. If sensitivity of the tests were the explanation of differences in the titer of a serum, the titer by one test must always be lower than by the other. The fact that the titers may sometimes be equal in both tests, sometimes higher by one method, and sometimes by the other, seems to exclude this idea of one test being more sensitive than the other.

An alternative explanation of these results could be that the indirect Coombs' test detects only, or mainly, one type or antibody, the fifth type, and that this type is not detected by the serum-albumin test. When agglutination is obtained by both the indirect Coombs' test and the serum-albumin test, there is a mixture of the fifth type with the antibodies detected by the albumin technique. Mohn and Witebsky's[12, 24, 25] discovery of an antibody in their

globulin fraction No. 3 which was detected by the Coombs' test and yet had no blocking properties and would not agglutinate the Rh-positive cells in the presence of normal adult human serum is also strong evidence for the suggestion that the Coombs' test may detect only one particular type of Rh antibody.

We realize, at least to some extent, the implication which this interpretation must have. First, the fifth type of antibody was previously regarded as a scientifically interesting, but very rare, antibody. Now one would have to regard it as one of the commonest types. Second, it might take over the role that was assigned in the earliest work to the saline agglutinins and later to the blocking antibodies, as the agent predominantly responsible for the clinical manifestations of erythroblastosis. This idea would be supported by clinical and laboratory evidence if the recent claims by Kelsall and Vos,[26] that there is a direct relationship between the indirect Coombs' titer and the severity of erythroblastosis, are confirmed by more extensive investigations by other workers. However, our results so far do not completely agree with their claims. We have noted that a high indirect Coombs' titer can be obtained from the serum of the mother of a normal or very mildly affected child. In addition, although a high titer of these antibodies has been found in the serum of mothers of infants so severely affected that they were stillborn or died shortly after birth, almost as many severely affected babies were born to mothers whose serum contained much smaller amounts of this antibody.

These results might be explained by assuming that the type of antibody detected by the indirect Coombs' test is a class of antibodies rather than a single entity. Hill and co-workers[19] have already suggested such a possibility in their paper on the separation of antibodies in different protein fractions by electrophoretic methods. Some forms of this new type of antibody may occur more often than others just as the Type 2 incomplete antibodies occur more frequently than the blocking antibodies and yet both of the latter are detected by the albumin test. The indirect Coombs' test would retain its value as an indication of the presence of the important form of antibody, but it might have to be supplemented by still other tests which would differentiate between its various components.

Highly speculative as some of these remarks are, they were made because, in the past, only the sensitivity of the methods was taken into account when discrepancies in results were observed. A review of our work as a whole strongly indicates that such differences are far better explained in terms of the preferential reaction of certain types of antibody in certain methods.

PART IV

INTERPRETATION OF THE RESULTS OBTAINED BY VARIOUS TECHNIQUES FOR ESTIMATION OF Rh ANTIBODIES

Diamond and Denton[5] stated that blocking antibodies (Type 1 incomplete antibodies in our nomenclature*) can be determined by the difference between the serum-albumin and the saline titers of the serum under investigation. In

*We have found it advisable to change the terms blocking for agglutinins, glutinins and blocking for glutinins which we used in our previous communication[27] and now designate them as Types 1, 2, and 3 incomplete antibodies.

view of the evidence presented in Parts II and III that the incomplete antibodies include three types, each with a different action, their statement cannot be accepted in its entirety.

In investigating any serum it is first necessary to determine which of the different forms of antibody are present. The results of investigations described previously have led to new ideas regarding the types of antibodies detected by albumin, indirect Coombs', and Wiener blocking tests. We suggest that the Wiener blocking test detects only Type 1 incomplete antibodies (blocking for agglutinins) and that the fifth type is the only one of the five known forms of antibody which is detected by the indirect Coombs' test (Table VIII).

TABLE VIII. TESTS FOR DETECTING AND DISTINGUISHING RH ANTIBODIES

TYPE OF Rh ANTIBODY	SERUM-ALBUMIN CONGLUTINATION TEST	INDIRECT COOMBS' TEST	SALINE AGGLUTINATION TEST	WIENER BLOCKING TEST
Complete antibodies				
Rh agglutinins	+	−	+	−
Incomplete antibodies detectable by cells in albumin				
1. Type 1 Blocking for agglutinins	+	−	−	+
2. Type 2 Nonblocking (probably the cryptagglutinoids)	+	−	−	−
3. Type 3 Blocking for agglutinins and Type 2 in serum-albumin	+	−	−	−
Fifth Type (Witebsky 4th type)	−	+	−	−

The three types of antibody detected by the serum-albumin technique can be distinguished in the following way: A comparison is made of the titers of Rh antibodies in two mixtures. The first consists of the serum under investigation (X) and a known serum containing saline agglutinins (A) or Type 2 incomplete antibodies (U_2); the second mixture contains similar proportions of the known serum and pooled homologous serum (P).

If the serum under investigation (X) contains any saline agglutinins, these must be destroyed by heat (56° C.).

A mixture of one part A or U_2 with two parts X appeared to be the most satisfactory proportion for mixtures which might contain significant amounts of blocking antibody for the following reasons:

1. With a dilution greater than one in three, an A of low titer might have such a weak agglutinating reaction that the effect of the blocking antibodies might not be observed.

2. With a dilution of less than one in three (that is, one in two) an X with a low titer of blocking antibodies might fail to cause a detectable blocking effect.

A mixture of one part A or U_2 with two parts of X is therefore recommended for these tests. The form of the antibody present in the serum under investigation can be then decided from the results set out in Table IX.

It is realized that it may be impossible to detect whether small amounts of Type 1 antibodies acting by adsorption are present in a particular serum, but if larger amounts are present they can be distinguished from Type 1 antibodies acting by neutralization as indicated previously.

If the types of antibody in a serum under investigation are known, the results of serum-albumin and saline titers may be interpreted as follows: (a) If Type 1 as well as Type 3 blocking antibodies are absent, the serum-albumin titer measures the concentration of Type 2 and saline agglutinins. In this instance, the difference between the albumin and saline titers gives the titer of the Type 2 incomplete antibodies. (b) If Type 3 blocking antibodies are absent but Type 1 present there are two possibilities, namely: (1) In a mixture containing Type 1 blocking antibodies acting by adsorption, the serum-albumin titer estimates the additive effect of saline agglutinins plus Types 1 and 2 incomplete antibodies.

TABLE IX

INCOMPLETE ANTIBODY IN THE SERUM UNDER INVESTIGATION	METHOD OF DISTINCTION		
1. Type 2 (if not blocked)	Saline titer of 1A + 2X	Equal to	Saline titer of 1A + 2P
2. Type 1 Blocking antibody for agglutinins by			
(a) preferential adsorption	If in sufficient concentration 1A + 2X will give no agglutination with one or more tubes and then exhibit prozone		
(b) competitive adsorption	If in sufficient concentration 1A + 2X will give some agglutination, but will exhibit prozone less marked than in (a)		
(c) neutralization	Saline titer of 1A + 2X	substantially less than	Saline titer of 1A + 2P
3. Type 3 Blocking antibody for Type 2 and agglutinins	Serum-albumin titer of $1U_2$ + 2X	substantially less than	Serum-albumin titer of $1U_2$ + 2P

Since the saline titer gives the total concentration of saline agglutinins, the difference between the serum-albumin and saline titers measures the concentration of Types 1 and 2 incomplete antibodies together. (2) If, however, the mixture contains Type 1 blocking antibodies acting by neutralization, the net result in saline will be a decrease in, or a complete elimination of, the saline agglutinins so that the saline titer measures excess agglutinins only. The difference between the serum-albumin and saline titers will give an erroneously high titer for Types 1 and 2 incomplete antibodies since "blocked" agglutinins will be included with them in the serum-albumin titer. (c) If Type 3 blocking antibodies are present, the serum-albumin titer represents the concentration of Type 3 and Type 1 antibodies only since both Type 2 and saline agglutinins are blocked by Type 3 in serum-albumin.

The common occurrence of the nonblocking Type 2 incomplete antibody is one of the main results stemming from the work described in these papers.

We have shown that it is these antibodies that are usually detected by Diamond and Denton's method. The latter estimates the blocking antibodies (our Type 1) in only the few sera containing no Type 2 antibodies.

The assumption that antibodies detected by the albumin method were all blocking antibodies has led to an unsolved problem. Blocking antibodies have been regarded as the major cause of erythroblastosis. However, it has become more and more obvious that there are many cases with high maternal albumin titers and minimal clinical effects. Our work may provide an answer to this problem.

SUMMARY

Part I:

1. Theories for the mode of action of blocking antibodies by (a) adsorption, which may be preferential or competitive, and (b) neutralization are discussed.
2. If the adsorption hypothesis is adopted, the saline titer in a mixture containing saline agglutinins as well as blocking antibodies represents the total Rh saline agglutinin concentration; if the neutralization theory is accepted, the saline titer in the mixture represents the excess of effective Rh saline agglutinins.

Part II:

1. Experiments to determine the mode of action of Rh antibodies are described.
2. These experiments led to the following conclusions:
   (a) Incomplete antibodies are not all of a type capable of blocking Rh agglutinins.
   (b) Incomplete antibodies detected by the albumin test include three types: Type 1, capable of blocking Rh saline agglutinins; Type 2, without blocking properties; and Type 3, eliminating the effect of Type 2 antibodies in albumin.

Part III:

1. Evidence for the existence of 5 types of Rh antibodies (Rh saline agglutinins and 4 other types) is examined in relation to the findings of other investigators.
2. In addition to the saline agglutinins and the three types of incomplete antibodies detected by the albumin technique, there is a fifth type detected only by the indirect Coombs' technique. This has already been reported by Hill, Haberman, and co-workers (cryptagglutinoid variant) as well as by Witebsky and Mohn (called, in their nomenclature, "4th type").
3. The indirect Coombs' test may be sensitive only, or mainly, to one type of antibody, the fifth type which is not detected by the albumin test.

Part IV:

In view of the conclusions in Parts I, II, and III, a new interpretation of the results obtained by various techniques for the estimation of Rh antibodies is suggested.

1. *The albumin titer.*
   a. The effect of incomplete antibodies and saline agglutinins is additive in albumin titrations.
   b. When the incomplete antibodies include no blocking antibodies, the difference between the saline and albumin titers gives the concentration of Type 2 incomplete antibodies.
   c. When blocking antibodies acting by neutralization are present in a serum, the saline titer estimates the excess saline agglutinins only.
2. *Wiener blocking test.*

   It seems probable that this test detects Type 1 incomplete antibodies, that is, blocking antibodies for saline agglutinins only.
3. *The indirect Coombs' test.*

   Evidence suggests that the fifth type of Rh antibody is the only one of the five known forms which is detected by the indirect Coombs' test.

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THE PRESERVATION OF BLOOD FOR TRANSFUSION

I. The Effect of Plastic Containers on Red Cells

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INTRODUCTION

THE use of plastic containers for collection of blood for transfusion has been recommended essentially because of physical properties. Among these may be listed lightness, resistance to breakage, nonwettable surfaces, and, consequently, improved platelet survival.[1]

Concerning the effect of the plastic containers on red cells, the reports in the literature are few and conflicting. Sack and associates,[5] using plastic containers, obtained results comparable with those obtained with the standard glass bottles. Muether and co-workers[6] and Knutson[7] likewise obtained satisfactory results with plastic bags. Wall and associates[8, 9] determined the changes in whole blood stored in the standard glass containers and in plastic containers, employing in vitro studies and determination of posttransfusion survival. Using blood from six donors, no evidence of excessive deterioration of red cells stored in plastic containers could be elicited by the in vitro tests. These authors found, however, that erythrocytes stored as whole blood in glass containers exhibited better posttransfusion survival than erythrocytes of blood stored in either of the two types of plastic containers. Nonagglutinable cell counts were used to determine the posttransfusion survival.

EXPERIMENTAL WORK

Our experience with plastic containers dates back to June, 1951. These preliminary studies gave variable results and, while the number of observations was not sufficiently large to warrant final conclusions, it appeared that the plastic containers did not improve the preservation of erythrocytes.[10]

During the months from January through March, 1952, plastic containers furnished by a different manufacturer were thoroughly tested. These containers and results of related studies will be designated as A-1952. Plastic containers from the same manufacturer were again tested during the months from January to April of 1953. The second series will be designated A-1953. In 1954, studies with plastic containers were resumed, using apparatus obtained from four laboratories. These will be designated as containers C, F, B, and A-1954, respectively. As controls, standard 650 ml. bottles of borosilicate glass were used.

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All containers used in 1952 and 1953 were filled with an isotonic low dextrose ACD solution[11]* and the blood was stored at 1° C. ± 0.5° C. Results obtained with plastic containers during these periods (A-1952 and A-1953) are therefore comparable.

All containers used in the 1954 studies were prepared with 75 ml. of the standard ACD solution (NIH formula A) with storage at 5° C. (± 1° C.). Results obtained during this period, therefore, are also comparable.

TECHNIQUE

The rate of collection of blood was maintained at 100 ml. per minute, and the collected blood was cooled to the temperature of storage in about one hour.

Hematocrit determinations were carried out by means of a high-speed centrifuge, employing capillary tubes.[12]

The red cell counts were carried out with standardized pipettes, counting at least 8 chambers.

The hemoglobin of the supernatant plasma was determined with a colorimetric benzidine method suggested by Gibson.[13] Hemoglobin reacts with benzidine and hydrogen peroxide, in the presence of a 20 per cent solution of acetic acid, with the production of a fairly stable port wine color, having a maximum absorption at 520 millimicrons. The intensity of this color is proportional to the hemoglobin concentration and may be measured in a photoelectric colorimeter.

The sugar was determined with the method of Nelson,[14] and the lactic acid determinations were carried out by the method of Barker and Summerson.[15]

The osmotic fragility curves were obtained with Parpart's method[16]; for sake of brevity only the amount of hemolysis at 0.63 per cent NaCl is noted in this report.

The determination of the adenosinetriphosphate (ATP) was carried out by precipitation of proteins with trichloracetic acid; ATP was then hydrolyzed (2 of 3 P) with sulfuric acid for 7 minutes at 100° C., and the phosphorus determined by the method of Gomori.[17]

The estimation of life span of transfused red cells by the Ashby method was carried out by transfusion of O blood in Group A recipients followed by agglutination of the A cells with dry anti-A rabbit sera,† and the count of 2,000 nonagglutinated cells. Standardization of the procedure included a mechanical device for uniform resuspension of cells.

The red cells were tagged with radioactive sodium chromate essentially by the method proposed by Ebaugh and co-workers.[18] Details of technique and calculations have been described separately.[19]

Blood volume determinations were carried out with the Evans blue dye method, using, for calculation of the body hematocrit, the formula:

venous hematocrit X 0.91.

RESULTS

Posttransfusion Survival Studies, 1952-1953.—Results of posttransfusion survival of erythrocytes collected and stored as whole blood in plastic bags A in 1952, using the nonagglutinable cell count, are shown in Fig. 1. The results indicate an average posttransfusion survival better than that obtained with conventional methods, such as glass bottles containing an ACD solution.

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		63 ml. contain:
*Trisodium citrate dihydrate	2.55 Gm.	1.6 Gm.
Citric acid monohydrate	0.80 Gm.	0.5 Gm.
Dextrose anhydrous	1.20 Gm.	0.756 Gm.
Water to 100 ml.		
pH 5.0		

63 ml. is used for 500 ml. of blood.

†Prepared by the Lederle Laboratories.

In view of our previous experience from the work carried out in 1951, it was considered likely, at the time, that the plastic containers themselves had little to do with the improved preservation and that the good results were attributed mainly to the lowering of the dextrose content of the ACD solution, the uniform controlled cooling, and the storage of blood at temperatures around 1° C.

Observations by the same technical staff, and employing exactly the same technique, were repeated in 1953 with containers furnished by the same manufacturer, but of a somewhat different design, so that they were readily distinguishable from the containers used in 1952.

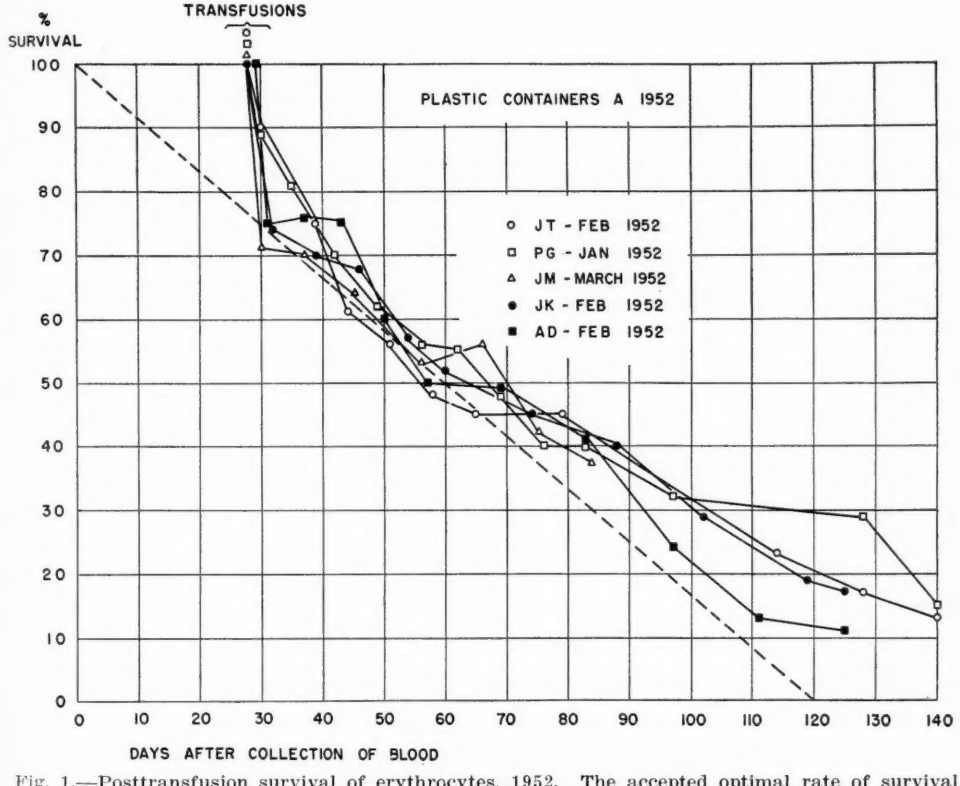

Fig. 1.—Posttransfusion survival of erythrocytes, 1952. The accepted optimal rate of survival of fresh, transfused blood is indicated by a dotted line.

The results of this second series of experiments, shown in Fig. 2, are evidently different from those obtained in 1952. The posttransfusion survival of red cells in this group of experiments is definitely worse than the average ob-obtained with the standard glass bottles.

In Vitro Studies, 1954.—All plastic bags C, F, B, and A-1954, as well as the standard glass bottles used in the more recent series of experiments, contained standard ACD formula NIH-A.

In vitro studies were carried out on all blood specimens after 21 days of storage and included the determination of the mean corpuscular volume, osmotic fragility, plasma hemoglobin, pH, dextrose, lactic acid, and ATP.

The average hemoglobin content of the supernatant plasma of blood collected and stored in the plastic containers F was found to be 237 mg. per cent, a value much higher than the average found in plasma of blood similarly stored in standard glass bottles (average 70 mg. per cent S. D. 17). The hemoglobin of plasma in the other plastic containers is significantly lower (average 30 mg. per cent) (Table I).

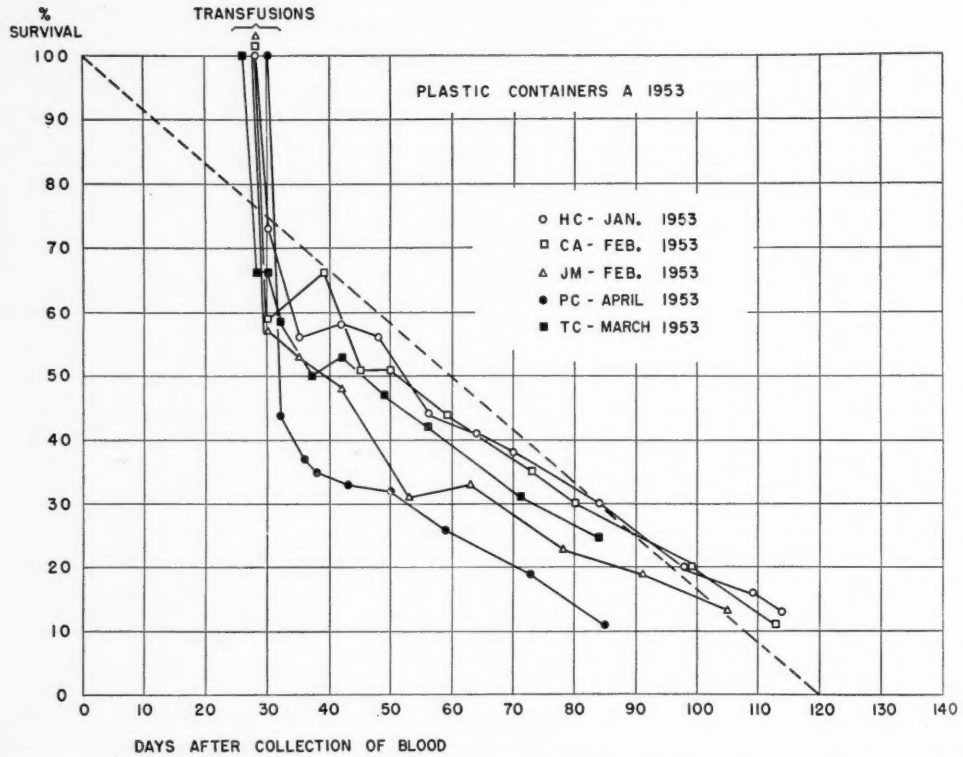

Fig. 2.—Posttransfusion survival of erythrocytes, 1953. The accepted optimal rate of survival of fresh, transfused blood is indicated by a dotted line.

Blood stored in one of the plastic bags (B) showed, as an average, a lower lactic acid and a higher dextrose content.

The ATP content of the blood stored in the plastic bags was found to be generally higher than in blood stored in glass containers. The average ATP content of blood specimens (22 in all) stored in plastic containers was 7.89 mg. per milliliter of packed red cells. For the blood stored in glass bottles, the value for the same determination was 5.72, with a S. D. = 1.43. The increase of the ATP values for the plastic containers appears suggestive. All other in vitro tests showed no significant differences (Table II).

Posttransfusion Survival Studies, 1954.—The posttransfusion survival studies were carried out with sodium chromate tagging. The results are graphically shown (Fig. 3). With plastic containers A-1954 and C, the re-

sults of both autotransfusions and transfusions into a compatible recipient show remarkably constant results. These results compare favorably with those obtained with the glass bottles.

The same is true of plastic containers B. If the single poor survival is not taken into consideration, the average survival extrapolated at 0 time is 82 instead of 78.

TABLE I

CONTAINERS	BLOOD LOT NO.	PLASMA HB. MG. PER CENT	AVERAGE
Plastic A	10796	26	34
	10797	23	
	A-5205	30	
	A-5211	15	
	10964	77	
Plastic B	10993	11	21
	B-4842	15	
	11015	37	
	A-5269	17	
	A-5276	27	
Plastic C	10392	23	33
	10429	78	
	10461	12	
	10478	15	
	10501	38	
Plastic F	10405	172	237
	10446	401	
	10465	141	
	10496	459	
	10526	102	
	10564	112	
	A-4927	269	
Glass Bottles	10404	76	70
	10451	47	S. D. 17
	10495	62	
	10584	82	
	10681	85	

All samples were stored at 5° C. for 21 days.

TABLE II

TYPE OF CONTAINER	NO. OF UNITS EXAM.	M. C. V.	PER CENT HEMOLYSIS IN 0.63 PER CENT NaCl	pH	DEXTROSE MG. PER CENT	LACTIC ACID MG. PER CENT	ATP
Plastic A-1954	5	98	3	6.72	306	119	7.94
Plastic B	5	97	4	6.69	382	89	7.47
Plastic C	5	95	3	6.71	308	113	8.48
Plastic F	7	98	6	6.68	266	113	7.74
Glass bottle	5	98	5	6.56	323	116	5.72

All samples were stored at 5° C. for 21 days.

Blood stored in the plastic containers F shows poorer posttransfusion survival of erythrocytes than that obtained with either the other plastic containers or the glass bottles. With standard borosilicate glass containers, the results are more scattered, the average being barely 70 per cent which is generally considered the lowest satisfactory.

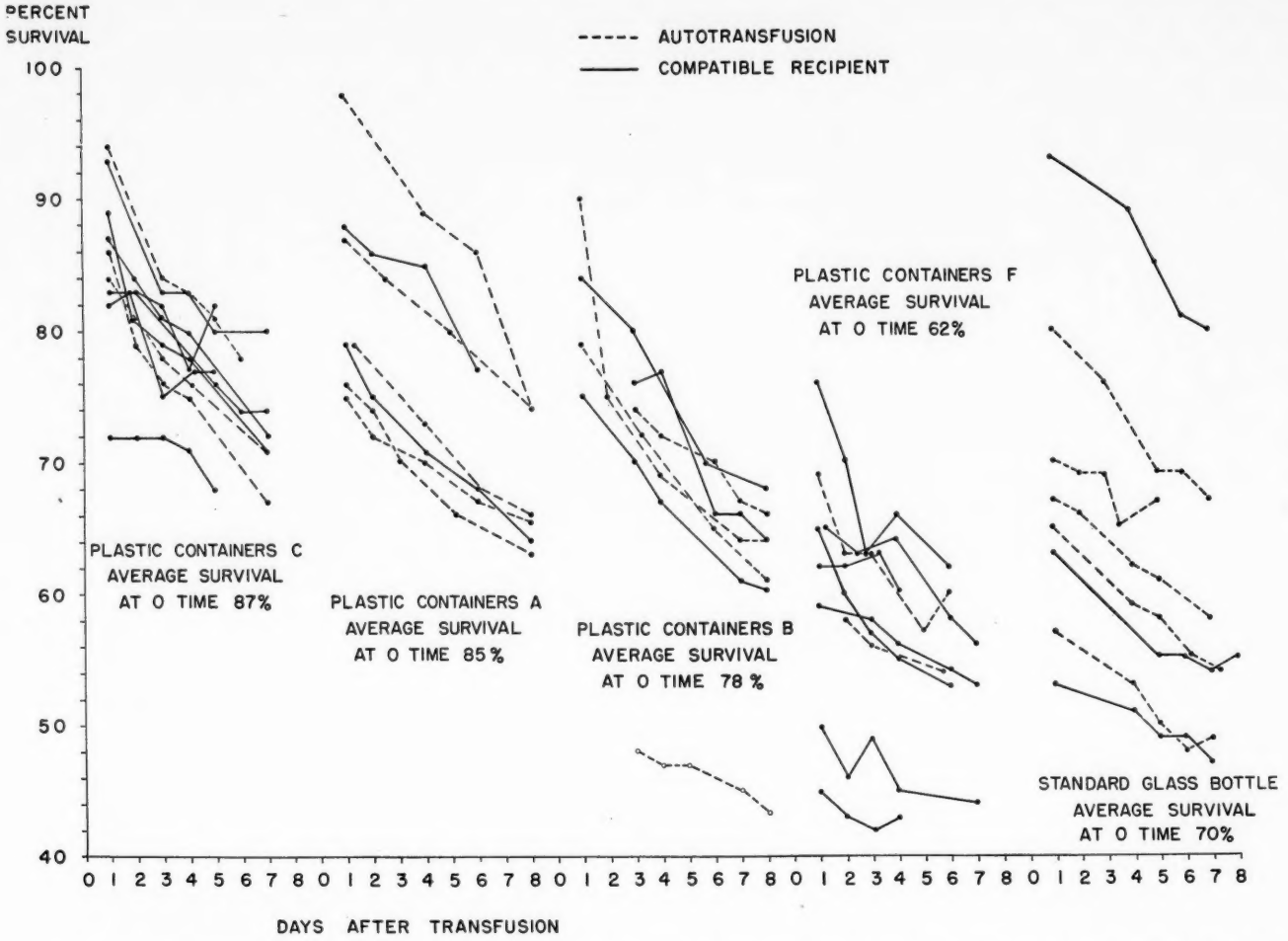
PERCENT SURVIVAL
AUTOTRANSFUSION
COMPATIBLE RECIPIENT
100
90
80
70
60
50
40
PLASTIC CONTAINERS C AVERAGE SURVIVAL AT 0 TIME 87%
PLASTIC CONTAINERS A AVERAGE SURVIVAL AT 0 TIME 85%
PLASTIC CONTAINERS B AVERAGE SURVIVAL AT 0 TIME 78%
PLASTIC CONTAINERS F AVERAGE SURVIVAL AT 0 TIME 62%
STANDARD GLASS BOTTLE AVERAGE SURVIVAL AT 0 TIME 70%
0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8
DAYS AFTER TRANSFUSION

Fig. 3.—Posttransfusion survival of erythrocytes, 1954. This figure shows the survival rate in 40 transfusions of red cells collected in ACD (NIH-A) solution and stored at 5° C. for 21 days. Fresh red cells tagged with radioactive sodium chromate and either autotransfused or transfused into a compatible recipient disappear from the blood stream during the first 10-day posttransfusion period at an average daily rate of 2 per cent.

The results obtained, suggest that under the experimental conditions no difference exists between autotransfusions and transfusions in compatible recipients. Whenever possible, each unit of stored blood was tested by both autotransfusion and transfusion into a compatible recipient.

The posttransfusion survival of red cells at 0 time was obtained by extrapolation of radioactivity data obtained 1 to 10 days posttransfusion. As already noted, the expected 100 per cent values were estimated from blood volume measurements with the Evans blue dye method and venous hematocrit X 0.91.

DISCUSSION

An analysis of experimental data obtained by in vitro studies and posttransfusion survival measurements of red cells stored as whole ACD blood in various types of plastic containers definitely suggests that these containers have a variable effect on the red cells. This effect varies not only with the source of plastic containers but also between successive lots of containers furnished by the same manufacturer.

This is not altogether surprising if one considers that the preparation of the plastic bags is subject to numerous variable factors, of which the nature of the original plastic material is but one. All containers tested in these studies were made of polyvinyl chloride. There have been numerous types of this material on the market, but lately the preparation among various producers has become fairly uniform, so that at present a more standardized and possibly purer material is available. However, several additions are necessary, according to the end use of the product, which may modify the properties of the basic material itself. To start with, the polyvinyl chloride is mixed with a fairly large amount of a plasticizer, often in a ratio as high as 2:1. A stabilizer also is needed, which may contain varying amounts of lead, calcium, cadmium, or barium. These stabilizers are particularly important in controlling thermoplastic properties; consequently consideration should be given to avoiding nonessential and too restricting specifications of thermoplastic properties for bags destined to contain blood, and subject therefore to relatively narrow limits of temperature variations. It is not unlikely that the stabilizers may contribute a great deal to the differences in the effect on the red cells stored in various types of plastic bags. Another factor which needs to be taken into consideration is the mold release agent. In addition, most manufacturers are now also adding a fungicide, which is often a mercurial compound. The addition of a fungicide is essential unless the outside of the plastic container is maintained sterile during the period of storage. Slow growth of molds on the surface of unprotected bags has been noted, which slowly diffuses through to the interior. Growth of molds on the surface of the plastic bag during the relatively short period of storage of blood has not been noted so far.

Finally, a variable factor may be introduced by the use of various detergents.

Fig. 3.—Posttransfusion survival of erythrocytes, 1954. This figure shows the survival rate in 40 transfusions of red cells collected in ACD (NIH-A) solution and stored at 5° C. for 21 days. Fresh red cells tagged with radioactive sodium chromate and either autotransfused or transfused into a compatible recipient disappear from the blood stream during the first 10-day posttransfusion period at an average daily rate of 2 per cent.

In our experience, it has been very difficult to obtain exact information concerning the nature of the plastic material, the plasticizers, the stabilizers, the mold release agents, fungicides, and detergents used in the preparation of the bags destined to contain blood. The manufacturers of the bags cannot control the production of the plastic itself, and the relatively small amount of material required for the preparation of bags to contain blood probably does not warrant, on the part of the manufacturers of the plastic material, production of a specific formula. Repeated inquiries, for instance, have failed to elicit any difference in the plastic used in the fabrication of containers labeled A-1952 and A-1953, although the effect on red cells of these two types of containers is apparently quite different. The only information given was that, for the preparation of the containers used in the 1953 series, a different detergent was used. It is interesting to note that insofar as we can determine, the containers used by Wall and co-workers[8, 9] and those used by us in the 1953 series (A-1953) were from the same lot, and poor results were obtained by both groups of investigators.

The high hemoglobin concentration in the plasma of blood stored in a type of plastic container (F) would suggest a primary toxic effect on the red cells. The posttransfusion survival studies confirm this observation.

Contrary to the opinion previously expressed,[10] it appears from the more recent and better controlled series of experiments (1954) that in some types of plastic containers the survival of red cells is actually better than when the red cells are stored in glass containers. This all-important point needs further study for definite verification and to determine the mechanism responsible for the improved preservation. Experimental work in this field is in progress.

It is at least interesting to speculate on the fact that the ATP content in blood stored 21 days in plastic containers is significantly higher than in similar units of blood stored in glass bottles.

In view of the fact that appraisal of the effect of the plastic material on the stored red cells requires the time-consuming determination of posttransfusion survival, it seems imperative that cooperation of producers of plastics and manufacturers of plastic equipment for blood transfusion be secured, so that a strict standardization of the final product may be obtained. These standardized containers could then be submitted to testing by posttransfusion survival studies before final release for general use.

The purpose of this investigation was essentially to determine the effect of the plastic water-repelling surfaces on the red blood cells stored as whole blood in an ACD solution, and no effort was made to evaluate the design and individual characteristics of the various bags, as most were only experimental models.

CONCLUSIONS

1. Plastic containers, available commercially or experimentally for storage of blood, exert variable effects on the red cells. Some containers appear to damage the red cells, reducing the posttransfusion survival rate; others

appear to have a definite beneficial effect on the posttransfusion survival of red cells. Such differences have been noted even within different lots of containers from the same manufacturer.

2. Before plastic containers can be recommended for general use for collection and storage of blood, it appears essential to obtain a rigid standardization, not only in the preparation of the basic material, polyvinyl chloride, but in all steps which modify the properties of the basic material, such as plasticizers, stabilizers, mold release agents, fungicides, and detergents.

Standardized containers should then be submitted to rigid experimental trials, including the effect of posttransfusion survival of red cells, before release for general use.

ADDENDUM

Posttransfusion survival studies of red cells stored in plastic bags furnished by company F were repeated recently. These bags were manufactured from a plastic of a composition different from that used previously. The results of these studies showed a good survival, similar to that noted with other good plastic bags.

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IN VITRO COMPARISON OF THE EFFECTS OF FOUR DIFFERENT PRESERVATIVE SOLUTIONS ON SINGLE DONOR BLOOD, WITH SPECIAL EMPHASIS ON RATE OF FLOW

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BETHESDA, MD.

SINCE the discoveries that the addition of glucose[1] and the lowering of pH[2] improved the preservation of stored blood, a variety of acid-citrate-dextrose solutions have been advanced for general use. The most commonly employed solutions are distinguishable by relatively small variations in the concentrations of the constituents and the volumes used per unit volume of donor blood. In vivo transfusion studies have not indicated that any of these is distinctly superior to the others, nor do they differ in ease of preparation or sterilization. Rapoport's[3] extensive studies showed only minor in vitro differences in single donor blood stored in a series of similar solutions available in 1947.

In view of this, it is difficult to choose among the currently available acid-citrate-dextrose solutions. Mechanical considerations play an important role in the selection—that is, chiefly volume relationships with regard to (a) size of the container, (b) volume of blood desired, and (c) ease of administration (i.e., rate of flow of final blood mixture). It is obvious that for a given volume infused, in order to achieve maximum sustained blood volume expansion, maximum rise in hemoglobin, and maximum plasma protein effect, dilution with preservative solution should be kept to a minimum. On the other hand, some workers[4] have stressed that unless relatively large volumes of anticoagulant solution are used, clotting or formation of fibrin flakes constitute a frequent problem, and the flow rate during transfusion is unduly prolonged.

The present study was undertaken to examine certain in vitro characteristics of single donor blood drawn into 4 currently advocated preservative solutions which illustrate the range of volume dilution encountered in general transfusion practice in the United States at the present time.

METHODS

The Solutions Examined.—The solutions shown in the accompanying table were prepared, and the volumes given in the last column were immediately sterilized in tightly sealed, rubber-stoppered glass containers having a total volume capacity of 175 ml. Sterilization was achieved by autoclaving for 30 minutes at 255° F. under 18 pounds pressure. No significant discoloration was noted.

The bottles had previously been calibrated with water and marked at the level equal to the volume of preservative solution plus 100 ml.

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	GM./100 ML. SOLUTION	ML. SOLUTION REQUIRED FOR 100 ML. BLOOD
NIH Solution A		
Trisodium citrate (dihydric) T.S.C.	2.2	
Citric acid (monohydric) C.A.	0.8	15
Dextrose (anhydrous) D.	2.45	
NIH Solution B		
T.S.C.	1.32	
C.A.	0.48	25
D.	1.47	
Bryn Mawr[5]		
T.S.C.	2.13	
C.A.	0.666	15
D.	2.0	
Strumia[6] *		
T.S.C.	2.54	
C.A.	0.793	12.6
D.	1.19	
C.-A.†		
T.S.C.	2.2	
C.A.	0.8	
D.	2.45	

*Designed for use only with blood stored at 1 to 2° C.

†Commercial-A (C-A) was obtained from commercial blood collection bottles said to contain NIH Solution A and was simply dispensed and immediately resterilized, as described previously.

The Donors and Blood Collection.—Blood was obtained from 3 normal men who fulfilled the usual criteria for satisfactory donors. Their blood hemoglobin concentrations were 15.0, 14.9, and 16.2 Gm. per cent, respectively. Each was bled by gravity, by use of a sterile vented system, 100 ml. being placed in each of the 5 bottles, as judged by filling to the mark on the glass described earlier. The bottles were agitated by gentle swirling during collection and were gently inverted 5 times at the completion. They were immediately placed in the storage refrigerator. Additional 5 ml. blood samples were drawn into tubes containing dry ''balanced'' oxalate mixture, to be used for measuring the hemoglobin concentration and hematocrit of each donor's undiluted blood.

Storage was at +4° to 6° C.

Sampling.—At periodic intervals during the ensuing month, each bottle was removed from the refrigerator, mixed by repeated inversion for 1 to 2 minutes, the rubber stopper sterilized with alcohol and iodine, and a 6 to 10 ml. sample removed with a dry sterile syringe.

Hemoglobin.—This was measured as oxyhemoglobin, employing duplicate 1/500 dilutions of each blood mixture in ammoniated water. The samples were read in an Evelyn colorimeter, employing a 540 mμ filter.

Hematocrits.—These were determined in duplicate, using standard Wintrobe hematocrit tubes 10 cm. in height, spun at 2,200 r.p.m. for 60 minutes in a centrifuge of radium 26 cm. No correction was made for trapped plasma.

Cell Swelling.—The cell swelling was determined by comparing the mean corpuscular hemoglobin concentration (M.C.H.C. = hemoglobin Gm. per cent/hematocrit per cent) of the stored samples with the M.C.H.C. of the donor's oxalated sample.

Plasma Hemoglobin.—This was determined by the method of Karr and Chornock,[7] modified in that: (a) dilutions of hemolyzed whole blood were used as a reference standard; (b) readings were made in a ''Lumetron'' photo colorimeter with a 650 mμ filter; and (c) a longer reaction time (40 to 45 minutes) in an ice bath was allowed to ensure that maximum coloration was produced.

Osmotic Fragility.—This was determined as percentage hemolysis in 0.6 per cent buffered saline solution after one hour's incubation at 24° to 25° C. (Parpart and coworkers[8]), omitting the use of complementary solutions.

Clotting.—The presence or absence of clots was determined by inspection at the time of each sampling and especially in the course of flow rate measurements.

Contamination.—Gram stains were made on smears from the settled plasma in each bottle at the end of the storage period. No organisms were seen in 14 of the 15 stored samples. A single plasma showed occasional gram-positive cocci, and it was concluded that insignificant bacterial multiplication had occurred in this sample.

Flow Rate.—The rate of flow of the five test mixtures was measured at the end of 30 to 31 days' storage by employing an Ostwald viscometer in a constant temperature water bath at 25° C. and comparing the results with distilled water tested under similar conditions.

Special Flow Rate Studies.—Additional studies were conducted to define the practical importance in clinical blood transfusion of the flow rate differences observed in the previous studies and to investigate the mechanism of those differences. Fresh (less than 1 week old) banked donor blood (500 ml. of blood plus 75 ml. NIH Solution A) was employed in these experiments, and standard transfusion equipment was used as a "clinical viscometer." Glass transfusion bottles containing 100 ml. of prefiltered blood, or other fluid to be tested, were fitted with a routine commercial blood recipient set, including a drip chamber with wire-mesh filter, plastic tubing, and plastic needle adapter. An 18 gauge needle was inserted into the glass airway tube, the bottle was hung inverted with the surface of the rubber stopper 32 inches above the mouth of a 10 ml. graduated cylinder, and the fluid mixture was allowed to flow into the cylinder through 1.5 inch long, 15, 18, 21, and 23 gauge hypodermic needles. All measurements were made at room temperature, which varied between 24° and 25° C.

The "flow time" required for 10 ml. of the mixture to be delivered from the outflow needle was measured by employing an electric timer accurate to within ± 0.5 second, and the results of 4 to 5 measurements were averaged to yield each final flow time result. The results were multiplied by 10 so that flow times could be expressed as "minutes per 100 ml. of blood."

RESULTS

General In Vitro Observations.—A summary of the results is shown in Table I.

Cell swelling: The degree of swelling was related, as expected, to the tonicity and the relative volume of the preservative solution and to the pH of the final mixtures. Thus, NIH Solution B, the most hypotonic, caused the cells to swell by 13 to 14 per cent of their original volume. NIH Solution A is close to isotonic, and swelling amounted to approximately 8 per cent, while Strumia's isotonic solution caused only about 5 per cent swelling. The swelling in the latter two mixtures was determined largely by the lowering of pH (down to 6.8 to 7.0), with consequent increase in the ionic equivalence of the hemoglobin. During storage there was no significant change in the percentage of swelling of the cells in any of the solutions as measured by progressive changes in M.C.H.C. All of these findings are in good agreement with those reported by Rapoport[3] for other similar A.C.D. solutions.

Spontaneous hemolysis: The absolute amounts of spontaneous hemolysis differed among the three donors, partly related to donor differences and partly to differences in technique in separating the supernatant plasmas. However, within each group, no consistent differences were noted in the amounts of

TABLE I. SUMMARY OF IN VITRO STUDIES ON SINGLE DONOR BLOODS STORED IN VARIOUS PRESERVATIVE SOLUTIONS

	UNITS		PRESERVATIVE SOLUTION				
			NIH A	NIH B	BRYN MAWR	STRUMIA	C-A
Cell swelling	%	Donor I	7	12	8	3	7
		Donor II	10	16	7	9	10
		Donor III	5	11	7	4	7
Spontaneous hemolysis: plasma hemoglobin after 8 to 10 days' storage	mg. %	Donor I	36	38	37	36	36
		Donor II	38	25	35	58	58
		Donor III	78	78	85	80	78
18 to 20 days' storage		Donor I	60	53	57	63	60
		Donor II	84	79	84	100	100
		Donor III	120	165	139	124	116
31 to 32 days' storage		Donor I	81	81	81	81	81
		Donor II	285	230	325	265	305
		Donor III	210	240	190	265	190
Hemolysis in 0.6 per cent buffered saline	%						
8 to 10 days' storage		Donor II	8	3	2	4	5
		Donor III	10	4	5	4	9
18 to 20 days' storage		Donor II	17	8	19	15	15
		Donor III	11	9	9	10	12
31 to 32 days' storage		Donor II	35	23	31	29	39
		Donor III	24	15	19	21	24

hemolysis occurring in the different preservative solutions. The fact that the values are somewhat higher than those usually reported is probably related to the repeated vigorous mixing attendant upon the periodic sampling procedures.

Fragility in 0.6 per cent buffered saline: No striking differences were noted among the five mixtures, although cells stored in NIH Solution B showed somewhat less hemolysis than cells stored in the other preservatives. The general range of the findings is in good agreement with Rapoport.[3]

Other Physical Characteristics.—

Clotting: No clots or detectable fibrin flakes were noted throughout the 31 to 32-day storage periods in any of the samples from the three donors.

TABLE II. COMPARISON OF FLOW TIMES OF SINGLE DONOR BLOOD AFTER 31 DAYS OF STORAGE IN VARIOUS PRESERVATIVE SOLUTIONS

PRESERVATIVE SOLUTION	FLOW TIME* (IN SECONDS)
Strumia	37.5
NIH Solution A	38.6
C-A	38.6
Bryn Mawr	36.6
NIH Solution B	32.6
Control—water	8.2

*Ostwald viscometer.

Flow rate: Some differences in flow rate were noted among the different mixtures tested in the Ostwald viscometer. Table II illustrates the results obtained with blood from Donor II. It is doubtful that any real differences in flow time were demonstrated in this experiment between the first four blood mixtures; however, blood stored in NIH Solution B had a distinctly shorter flow time than the others.

These measurements were made in a small viscometer, employing a small volume of blood and determining the flow over a relatively long course (11.5 cm.) through a capillary glass tube of narrow bore (1 mm.). It was thought interesting to extend these studies employing routine transfusion equipment as a "clinical viscometer" as described below.

Special flow rate studies: Among the most important factors determining the rate of flow of blood during the administration of a transfusion are (a) the differential between the pressure applied to the blood and the opposing pressure in the vein into which it is flowing, (b) the length and diameter of the narrowest segment of tubular pathway through which it flows, (c) the proportion of red cells present, and (d) the nature of their suspending fluid.

Pressure: Studies in the "clinical viscometer" employing blood columns of 48, 42, and 36 inches in height and with a 21 gauge outflow needle confirmed that the flow time varies in a linear fashion inversely with the pressure. In the remaining experiments in which transfusion equipment was used, a standard applied pressure was employed throughout, the height of 32 inches (see Methods) having been selected as one easily achieved and commonly employed in transfusion practice. No attempt was made to apply a standard opposing pressure at the point of outflow.

Internal diameter of needle: The diameters of the various components of the recipient set are large relative to the internal diameters of the outflow needles. Thus, in the "clinical viscometer," for a given test fluid under a given applied pressure, the flow rate will be largely determined by the internal diameter of the needle employed and will be dependent very little upon small variations in the characteristics of the recipient set. For blood mixtures with three different hemoglobin concentrations, the relationship of flow time to internal needle diameter is shown in Table III. The graphic presentation of the same data in Fig. 1 emphasizes the striking increase in flow time which results when needles of smaller bore are employed.

TABLE III. RELATIONSHIP OF FLOW TIME TO NEEDLE GAUGE

NEEDLE GAUGE	INTERNAL DIAMETER (MM.)	FLOW TIME FOR 100 ML. OF BLOOD (MINUTES)		
		HGB. 9.8 GM. %	HGB. 12.6 GM. %	HGB. 14.3 GM. %
15*	1.372	1.0	1.2	1.2
16	1.194	1.5	2.1	2.4
17	1.068	2.0	2.7	3.4
18*	0.838	2.7	3.6	4.3
19	0.686	6.0	7.6	9.0
20	0.594	9.5	11.9	13.9
21*	0.508	13.3	16.0	18.6
22	0.406	34.5	42.7	50.8
23*	0.330	56.5	70.0	83.8

*Flow times measured, as described under Methods. Flow times for needles of gauge 16, 17, 19, 20, and 22 are estimated from the curves in Fig. 1.

Relative volume of preservative solution: The relative volume of preservative solution employed per unit volume of stored blood will affect by dilution both the proportion of red cells present and the nature of their suspending

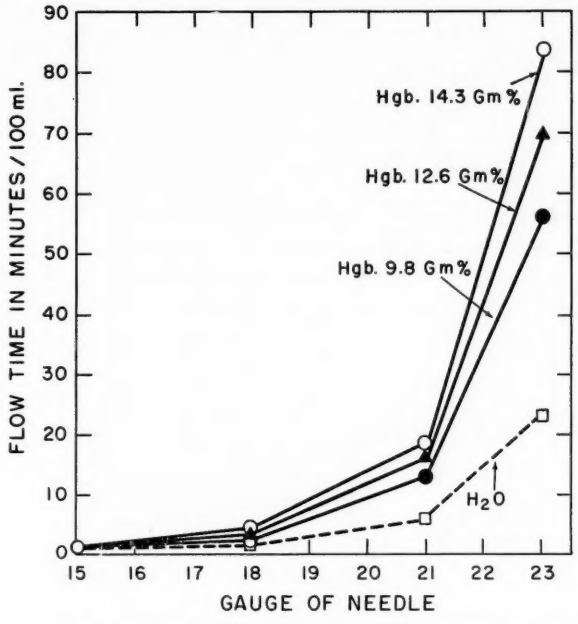

Fig. 1.—Relationship of flow time to needle gauge.

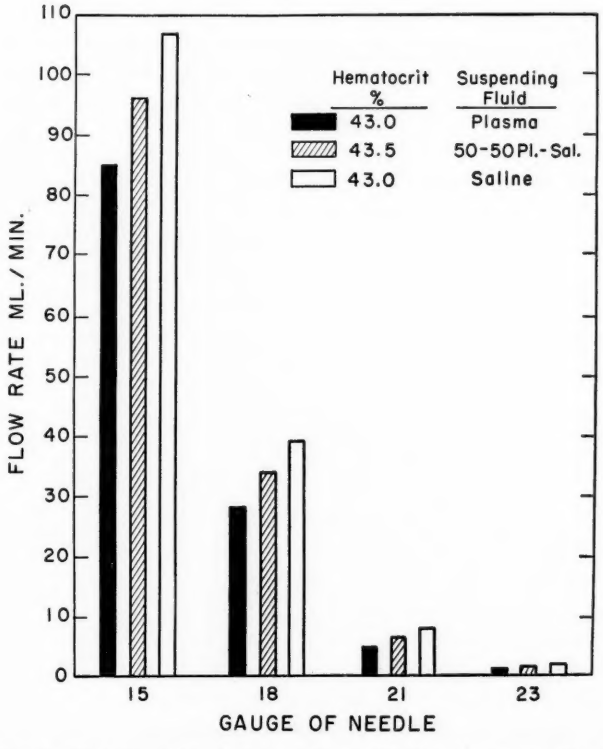

Fig. 2.—Relationship of flow rate to dilution of suspending plasma.

medium. Because the various preservative solutions are acid (pH 5.0) and vary as to their tonicity (most of them being slightly to moderately hypotonic), there results a shift of some fluid out of the plasma-anticoagulant phase into the interior of the cells. Thus, the degree of dilution of the plasma is always slightly less and the hematocrit of the final mixture somewhat higher than would be predicted by simple mathematical calculation.

Effect of dilution of plasma: To define the effect of the dilution of plasma by an isotonic watery solution, two aliquots of fresh banked blood (stored in NIH Solution A) were centrifuged, all the plasma removed and replaced with (a) an equal volume of 50 per cent solution plasma in isotonic saline and (b) an equal volume of saline solution. The flow rates of these mixtures were compared with that of the original blood, with the results indicated in Fig. 2. The proportion of cells present was the same in each mixture, and the observed differences in flow rate can be attributed to the effect of diluting the plasma. From these data, it can be computed that for every 10 per cent increase in dilution of the plasma there will be an increase not exceeding 4 per cent in the flow rate of the red cell suspension.

Effect of dilution of the total number of red cells: The volume of preservative solution employed will produce a predictable lowering in the total hemoglobin concentration of the final mixture. However, as mentioned previously, some preservative solutions cause more red cell swelling than others, and upon this factor will depend the final hematocrit of the mixture. It was important to establish whether rate of flow is dependent more closely upon the hemoglobin concentration of the sample (representative of the total number of red cells present) or upon the hematocrit (that is, total volume occupied by red cells).

Fresh red cells from a single donor were separated into three aliquots which were washed 4 times in buffered (pH 7.5) 0.6 per cent, 0.9 per cent, and 1.2 per cent sodium chloride solutions, respectively. The cells were finally resuspended in the buffered solutions so as to have a final hematocrit of 43 per cent. The effect of washing with solutions of different tonicity upon the total hemoglobin concentration of the three mixtures is shown in the third column of Table IV.

TABLE IV. DEPENDENCE OF FLOW TIME ON NUMBER OF CELLS PRESENT I. HEMOGLOBIN VARIED AND HEMATOCRIT CONSTANT

BUFFERED SALINE WASH SOLUTION %	HEMATOCRIT %	HEMOGLOBIN GM. %	M.C.H.C. %	FLOW TIME IN MINUTES FOR 100 ML.			
				15 GAUGE	18	21	23
0.6	43.2	12.0	27.8	0.89	2.38	12.4	47.8
0.9	43.0	14.7	34.1	1.03	2.74	14.6	57.2
1.2	42.9	16.6	38.7	1.38	3.48	19 4	75.8

It is evident, most clearly shown in the values obtained using the small-caliber needles, that the flow times are markedly affected by the hemoglobin levels (60 per cent difference between flow times at the extremes of the range studied). A second experiment was then performed in which suspensions of

the same three groups of washed cells were made up to identical hemoglobin values; the magnitude of the resultant hematocrit differences are seen in the second column of Table V.

TABLE V. DEPENDENCE OF FLOW TIME ON NUMBER OF CELLS PRESENT II. HEMATOCRIT VARIED AND HEMOGLOBIN CONSTANT

BUFFERED SALINE WASH SOLUTION %	HEMATOCRIT %	HEMOGLOBIN GM. %	M.C.H.C. %	FLOW TIME IN MINUTES FOR 100 ML.			
				15 GAUGE	18	21	23
0.6	43.2	12.0	27.8	0.89	2.38	12.4	47.8
0.9	35.0	12.0	34.3	0.92	2.38	12.3	47.8
1.2	30.9	12.0	38.9	0.92	2.5	13.4	51.9

It can be seen that the flow times obtained with these mixtures showed scarcely detectable differences, the maximum variation being 8.5 per cent between the extremes observed. Thus, it is evident that in evaluating the effect of the volume of anticoagulant solution employed, the final hemoglobin concentration of the sample will be a more important determinant of flow time than the final hematocrit value achieved.

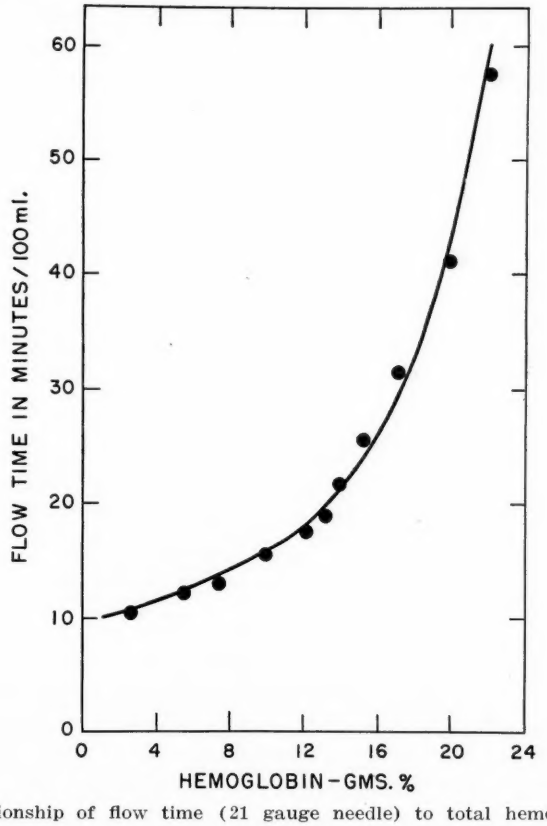

Fig. 3.—Relationship of flow time (21 gauge needle) to total hemoglobin concentration.

In order to define the relationship of flow time to total hemoglobin concentration, mixtures of red cells in their own plasma were made up, covering the hemoglobin range from 2.6 to 21.8 Gm. per cent. The flow times are il-

lustrated in Fig. 3. It can be shown that over the hemoglobin range routinely encountered in stored blood (10 to 15 Gm. per cent), there is approximately 10 per cent increase in flow time for every 1.0 Gm. per cent rise in hemoglobin concentration.

Combined dilution effects: By reference to the recommended volumes for the various anticoagulant solutions studies in this report (see Methods), it can be calculated that for a given donor blood sample, the maximum range of variation in final plasma and hemoglobin concentrations resulting from the choice of solution will not exceed 10 per cent and 1.3 Gm. per cent, respectively. The combined effects of these two factors can be expected to produce a difference in flow time of approximately 15 per cent, the more rapid flow rate being achieved when the largest volume (Solution B) is employed. However, when Solution B is used, the total volume infused into the patient per unit volume of blood is 10 per cent greater than for an equivalent volume of blood stored in the Strumia solution. Thus, the use of the larger volume of anticoagulant solution results in a net shortening of flow time of only 5 per cent per unit volume of donor blood. The practical importance of this difference in flow time for 500 ml. of donor blood (hemoglobin 15 Gm. per cent) is small, as illustrated in Table VI.

TABLE VI. EFFECT OF VOLUME OF PRESERVATIVE SOLUTION ON FLOW TIME FOR 500 ML. OF DONOR BLOOD

NEEDLE GAUGE	NIH SOLUTION B 125 ML./500 ML. WHOLE BLOOD (FINAL HGB. 12.0 GM. %) (MINUTES)	STRUMIA 63 ML./500 ML. WHOLE BLOOD (FINAL HGB. 13.3 GM. %) (MINUTES)
15	6.75	7.09
18	21.0	22.05
21	115.0	120.75
23	433.0	454.7

DISCUSSION

The results of these limited studies confirm that there is very little in vitro difference between bloods stored in the four preservative solutions which have been examined. Clotting was not a problem, and special studies of the factors determining flow time showed that the differences to be expected from choice among the solutions will be small and of negligible importance under conditions generally encountered in transfusion practice.

It should be stressed that no attempt was made in the present study to evaluate the importance of the relative volumes of the anticoagulant solutions in protecting against clot formation when mixing is inadequate. Another problem not studied is the frequency of clot formation in bloods collected by the vacuum method as compared to collection by gravity. It is worth pointing out that since the anticoagulant solution is of relatively low specific gravity, it will tend to overlie the blood if mixing is neglected.

The major cause of difficulty in the administration of stored blood is improper mixing. Mixing is important at two stages: (a) at the time of blood

collection, when failure to mix adequately allows some fibrin formation to occur (the amount of citrate ion present in commonly used A.C.D. solutions is at least double that necessary to bind available calcium ion); and (b) at the time of administration, when inadequate mixing results in the transfusion of a relatively high hematocrit suspension during the first half of the transfusion. To secure adequate mixing on both of these occasions does not require extraordinary expenditure of time or effort. Two or three times during an ordinary collection of 500 ml. of blood the bottle should be gently swirled, and at the conclusion of the bleeding, after the bottle is disconnected from the donor set, it should be inverted 4 to 5 times. These maneuvers will ensure against fibrin precipitation. At the time of administration, the bottle should be sharply inverted 10 to 12 times prior to insertion of the recipient set and airway needle. If these procedures are conscientiously performed, the ease of administration of blood drawn into any of these preservative solutions should be assured.

The present studies have not provided any in vitro evidence favoring the selection of any one of the four solutions which were investigated. In view of the general desirability of transfusing a product which as nearly as possible resembles "whole blood," it would seem economical from the point of view both of the patient and of storage space in the blood bank to select a solution giving satisfactory preservation for the least volume of solution required.

SUMMARY

1. Single donor blood from three normal donors was stored in four currently advocated A.C.D. solutions.

2. Observations on cell swelling, spontaneous hemolysis, osmotic fragility, and flow rate revealed practically no important differences among the four mixtures.

3. Special studies are described employing transfusion equipment as a "clinical viscometer" and indicating the ways in which differences in flow time result from the dilution effect of the anticoagulant solution.

4. Differences in the flow time of blood drawn into the four solutions studies in this investigation were shown to be of negligible importance under routine transfusion conditions.

5. Emphasis was placed upon the importance of adequate mixing during blood collection and prior to administration, in facilitating the ease of administration of all four mixtures.

6. No evaluation was made of the importance of the relative volumes of the anticoagulant solutions nor of bleeding by the vacuum versus the gravity method in protecting against clotting, when mixing during blood collection is inadequate.

We are indebted to Mr. Robert W. Kolb for performing the plasma hemoglobin determinations and to Mrs. Ida Yee for the flow time results described in Table II. We are especially grateful to Dr. J. T. Tripp for his advice and encouragement.

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AN UNUSUAL ANTIBODY PATTERN IN A CASE OF IDIOPATHIC ACQUIRED HEMOLYTIC ANEMIA

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ANTIBODIES reacting with erythrocytes have long been known to be remarkably diverse in behavior. Among the variations encountered have been differences in thermal amplitude, agglutinating, or hemolyzing ability, and reaction in various media. In different sera many of these variations have been combined. Recently Dacie and de Gruchy[1] emphasized the differences in antibody patterns found in acquired hemolytic anemia. When these investigators obtained an antibody profile by means of a battery of tests (direct and indirect Coombs', warm and cold agglutinins, hemolysins, agglutinins from eluates of whole erythrocytes or of stroma, and agglutinins for enzyme-treated cells), the patterns which appeared were highly diversified.

A pattern in which agglutinin activity was directed against enzyme-treated cells alone has been reported in only three publications. In 1952, Dausset[2] referred briefly to four cases of acquired hemolytic anemia in which the direct and indirect antiglobulin tests were negative but in which only the trypsinized cell reaction was positive during the entire course of the disease. Later Dacie and Cutbush[3] reported on the specificity of the antibodies from 10 patients with acquired hemolytic anemia. In some, the antibody obtained either from the serum or from an eluate of coated erythrocytes acted best or only with trypsin-treated cells. Another somewhat similar case was described by Dacie.[4] During remission, the direct and indirect antiglobulin tests became negative, but the patient was left with a nonspecific high-titer antibody in his serum which agglutinated trypsinized corpuscles.

The present report concerns a patient with an unusual type of idiopathic acquired hemolytic anemia. In contrast to the usual cases, the presenting symptom was that of hemoglobinuria. Moreover, the patient's serum contained a potent agglutinin for enzyme-treated red cells, not associated with a positive direct Coombs' test. Detailed studies of the patient's agglutinin were undertaken and showed certain similarities to and differences from the normal autoagglutinin active with trypsin-treated erythrocytes.

CASE REPORT

M. N. was a 59-year-old white woman whose present illness dated from 1946. At that time she had had an episode of abdominal pain and jaundice for which a cholecystectomy was performed. She was then well until September, 1951, when, during a period of three weeks, she experienced three attacks of chills, nausea, vomiting, and slight jaundice,

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but no abdominal pain. Each attack was of about twenty-four hours' duration. The past and family history was not contributory. There was no history of exposure to fava bean or any other known hemolytic agent. During the third attack she consulted her physician, who found a definite icteric tinge to the skin, a firm liver edge palpable 3 cm. below the right costal margin, and a blood pressure of 160/104. The spleen was not palpable and there were no other significant physical findings. The hemoglobin was 7.8 Gm. per 100 ml., erythrocytes 2.85 million per cubic millimeter, icterus index 18 units, and reticulocytes 3.2 per cent. Erythrocyte osmotic fragility and liver function studies were within normal limits, as were roentgenograms of the chest and intestinal tract. During the three weeks following the third episode, the patient was asymptomatic and there was a progressive spontaneous increase in hemoglobin to 9.6 Gm. per 100 ml., and of erythrocytes to 3.19 million. At this time, the following additional findings were obtained: the Coombs' test was negative, and the fecal urobilinogen output was 25 mg. per day. White blood cells and platelets were within normal limits, but the bone marrow showed marked erythroid hyperplasia. A red cell survival time performed by the Ashby technique, using two units of cell mass, showed the average cell life to be about 45 days. Following this transfusion, the hemoglobin rose to 13.3 Gm. per 100 ml. and the erythrocytes to 4.71 million per cubic millimeter. After 36 days, the hemoglobin had dropped to 12.2 Gm. per 100 ml. and the erythrocytes to 4.06 million.

For the next year the patient remained well and no further progress of her anemia was noted. She then began to have attacks every two or three months, each lasting two or three days, during which she noted a feeling of constriction in the chest and the urine was dark. In January, 1954, a similar attack occurred but this time the urine was described as "bloody." Urinalysis was reported as showing pyuria, and Aureomycin therapy was instituted. Although the symptoms disappeared in a few days and the urine returned to its normal color promptly, the pyuria continued.

On March 6, 1954, the patient developed an acute onset of substernal and low-back pain. Clinically, she was in shock, and her urine was the color of port wine. The following day she went into acute renal insufficiency, and continued virtually anuric for the next 10 days. The nonprotein nitrogen rose to 257 mg. per cent and the creatinine to 13 mg. per cent. She gradually recovered from this episode on conservative therapy, and renal function returned to normal. A subsequent complete urologic examination was normal. At the onset of the episode, the icterus index was 47 units, Coombs' test negative, hemoglobin 14.1 Gm. per 100 ml., erythrocytes 4.9 million per cubic millimeter, and leukocytes 4,850 per cubic millimeter with a normal differential count. The urine was found to contain a few erythrocytes, but was strongly guaiac and benzidine positive. Urine culture showed *Proteus vulgaris.* Two days after onset, the hemoglobin had dropped to 11.2 Gm. per 100 ml. and the erythrocyte count to 4.46 million per cubic millimeter. The leukocyte count rose to 22,000 per cubic millimeter with 97 per cent neutrophils. One month later, the hemoglobin was 11.95 Gm. per 100 ml., the erythrocytes 3.2 million per cubic millimeter, reticulocytes 6.1 per cent, leukocytes 4,200 per cubic millimeter with a normal differential count, and the fecal urobilinogen 95 mg. per day. The bone marrow at this time showed moderate erythroid hyperplasia. The Ham and Crosby test for paroxysmal nocturnal hemoglobinuria, the Donath-Landsteiner test for cold-warm hemolysin, and routine studies for serum hemolysin were negative. Cortisone in a daily dosage of 75 mg. was started at this time. During the following two months, there was a gradual return of the hemoglobin to 14.9 Gm. per 100 ml., the erythrocytes to 4.77 million per cubic millimeter, and the reticulocytes to 1.5 per cent. The blood count had remained normal when the patient was last seen on Oct. 11, 1954. During this period the patient had experienced several transient episodes of chilliness and headache, but no jaundice or hemoglobinuria had occurred. The dosage of cortisone had been gradually reduced to 12.5 mg. per day.

EXPERIMENTAL

I. *Patient's Erythrocytes.*—

Direct Coombs' test: Serial dilutions of a Coombs' reagent prepared in this laboratory were made up in saline solution. An equal volume of a 2 per cent saline suspension of the patient's washed red cells was mixed with 0.05 ml. of each dilution. Both untreated and trypsinized cells were used. The mixture was allowed to incubate at room temperature for 30 minutes and then lightly centrifuged before reading.

Indirect Coombs' test: A quantity of 0.025 ml. of normal packed red cells, previously washed 3 times in isotonic saline solution, was incubated in 1 ml. of erythrocyte stroma eluates, or in 1 ml. of patient's serum for 2 hours at 37° C. Following this sensitization process, the cells were again washed in saline solution, as above, and tested as in the direct test.

Trypsin-treated cells: A stock solution of crystalline trypsin (Tryptar) was prepared by dissolving 250 mg. of "tryptic activity" in 25 ml. of N/20 HCl. This preparation was stable when stored at 4° C. Prior to use it was diluted 1-10 in M/15 phosphate buffer of pH 7.8. One volume of washed red blood cells was mixed with 4 volumes of diluted trypsin and incubated at 37° C. for one hour. Following treatment with the enzyme, the cells were again washed 3 times and then suspended in saline solution to a final concentration of about 2 per cent.

Albumin test: Two drops of Armour's 30 per cent bovine albumin were added to a 2 per cent serum suspension of the patient's cells. The mixture was centrifuged immediately, examined, then incubated at room temperature for an hour, centrifuged again, and read for agglutination.

Red cell eluate: The method was that described by van Loghem and van der Hart,[5] which involves heating washed red cells in saline solution at 56° C. and separating the eluate. This eluate was tested for its ability to agglutinate trypsin-treated cells or to render untreated cells Coombs' positive.

Red cell stroma eluate: Preparation followed the methods of van Loghem and van der Hart.[5] After lysis in distilled water, the stroma was precipitated by reduction of pH to 5.6-5.8. Washed stroma was then eluted and studied in the same manner as with the red cell eluate.

Results.—The patient was Group A_1M Rh positive, genotype cDE/cde. The untreated erythrocytes from numerous blood samples drawn during a period of several months were consistently Coombs' negative. Three separately prepared antiglobulin sera were employed in all dilutions up to 1-640, thus ruling out a possible prozone phenomenon. Immediate centrifugation of the mixture of cell and antiglobulin serum did not elicit a positive reaction, although this technique had on occasion been successful when incubation had yielded negative results with certain other patients. On the assumption that the receptor might be unusually "deep" in the red cell, the patient's cells were first treated with trypsin and then tested with the Coombs' reagent. No agglutination resulted.

Since some antibody-coated cells may agglutinate in albumin and yet be Coombs' negative, our patient's cells were subjected to the albumin test. On immediate centrifugation, there was no agglutination. However, when the patient's cells were suspended in albumin and incubated in a drop of her own serum, agglutination of the cells was observed. This positive albumin test was only occasionally present.

An attempt was made to concentrate possible antibody coating the red cell by preparation of eluates from large amounts of red cells. Van Loghem and van der Hart[5] found this technique effective when the direct Coombs' test was positive. Eluates prepared from red cells and from red cell stroma of the patient failed to sensitize untreated or trypsinized red cells even when eluates prepared from a packed volume of 1 ml. of patient's cells were tested against minute quantities of normal cells.

II. *Patient's Serum.*—

Agglutination titers: Doubling serial dilutions of the serum were prepared in saline solution, and, to each 0.05 ml. of serum dilution, an equal volume of appropriate cell suspension was added. Immediately after mixing or after incubation, the tube of serum-cell mixture was lightly centrifuged and examined both grossly and microscopically. Mixtures incubated at refrigerator temperature were read without centrifugation.

Modified erythrocytes: Cells that were washed 3 times in saline solution were employed in all instances. Papain-treated cells were prepared according to the method of Hoyt and Zwicker.[6] Cells were treated with periodate according to the method of Stewart.[7]

Results.—The serum contained a highly active agglutinin (titer 1-624 at 37° C.) affecting trypsin-treated cells. As is seen in Table I, the agglutinin was relatively unaffected by temperature. If the serum and cells were centrifuged immediately following mixing, the titer was slightly lower. The agglutinin behaved as a panagglutinin for trypsinized cells with no significant difference in titer for Groups O, A_1, or A_2 cells.

TABLE I. AGGLUTINATION REACTIONS OF THE PATIENT'S SERUM ILLUSTRATING EFFECT OF TEMPERATURE AND TYPE OF ERYTHROCYTE

TYPE OF RED CELL	TEMPERATURE (C.)	AGGLUTINATION TITER
Patient's trypsinized cell	4°	1-512
Patient's trypsinized cell	20°	1-512
Patient's trypsinized cell	37°	1-1,024
Patient's untreated cell	4°	1-16
Group A_1 trypsinized cell	20°	1-256
Group A_2 trypsinized cell	20°	1-256
Group O trypsinized cell	37°	1-512
Group A_1 papainized cell	20°	1-64
Group A_1 papainized cell	37°	1-32
Group A_1 untreated cell	20°	0
Group A_2 untreated cell	20°	0
Group B untreated cell	20°	1-64

This serum autoagglutinin was not active against untreated homologous erythrocytes. The indirect Coombs' test was negative, whether cells of group A_1Rh negative, A_1Rh positive, O Rh negative, or O Rh positive were used. No complete or incomplete Rh antibodies were detected in saline solution or albumin dilutions of the serum and cells.*

The anti-B isoagglutinin titer was 1-64, and the cold agglutinin titer was 1-16 against untreated cells. Both were within the normal range.

*The findings on this patient were confirmed by Dr. Philip Levine of the Ortho Research Foundation. The patient was reported as Group A, Rh positive, type R_2r. Direct Coombs' test was negative. The serum was tested with a panel of selected bloods in saline solution followed by the indirect Coombs' test and there were no atypical reactions. On testing the serum with trypsinized cells, all types were agglutinated. In Dr. Levine's opinion, the antibody was nonspecific and more active at the lower temperatures than at 37° C.

It was also found that the patient's serum agglutinated papain-treated cells. The titer was 1-64; whereas normal sera were incapable of agglutinating such cells. The difference in titer between papain- and trypsin-treated erythrocytes could represent technical rather than qualitative differences. It is possible that papain produced a lesser degree of cell alteration than that achieved by trypsin.

Periodate treatment of erythrocytes renders them panagglutinable by human sera, and it also exposes receptors for viruses. Periodate-treated cells reacted identically to normal and to our patient's serum.

The addition of complement in the form of fresh human serum in no instance produced hemolysis of the trypsinized agglutinated cells of the patient, or of normal cells treated with the patient's serum.

III. *Properties of the Agglutinin.*—

Storage of the serum for three months at –20° C. did not decrease the agglutinin titer of the serum for trypsinized cells.

The patient's agglutinin showed moderate heat lability. It was partially inactivated by a temperature of 62° C. and completely destroyed by heating to 65° C. for 10 minutes. The normal serum autoagglutinin[8] seemed to be slightly more heat labile, but the differences obtained may reflect differences in initial titer. The data are shown in Table II, where it can also be seen that the patient's heated serum developed a prozone, a phenomenon which could be repeatedly demonstrated. The unagglutinated cells in the first few tubes appeared to be coated with agglutinin, for when the cells were washed in saline solution, spontaneous agglutination occurred. The present agglutinin is more heat labile than those responsible for the indirect Coombs' test in patients with acquired hemolytic anemia. In this laboratory, the latter have shown no loss of activity up to temperatures which coagulate serum.

TABLE II. EFFECT OF HEAT ON NORMAL AND PATIENT'S AUTOAGGLUTININ TITER FOR TRYPSINIZED ERYTHROCYTES

	DILUTIONS OF SERUM IN SALINE SOLUTION									
	1-1	1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512
Patient's Serum	*Degree of Agglutination*									
Unheated control	s	s	s	s	s	s	s	s	3	–
Heated 62° C.	–	–	–	3	4	4	3	1	–	–
Heated 65° C.	–	–	–	–	–	–	–	–	–	–
Normal Serum										
Unheated control	s	s	4	2	–	–				
Heated 59° C.	4	4	2	1	–	–				
Heated 62° C.	–	–	–	–	–	–				

s = solid mass of agglutinated cells.

The electrophoretic mobility of the patient's agglutinin was determined by the method described by Payne and Deming.[9] The basis of this method is elution of segments cut from a paper electrophoretic pattern. The agglutinin migrated with the gamma globulins of greatest mobility. The same segment also contained the patient's β isoagglutinin and is the usual site for other normal α and β isoagglutinins. The stained paper electrophoretic pattern showed normal protein distribution.

IV. *Agglutination Reversal.*—

The patient's agglutinin resembled the normal autoagglutinin of Rosenthal and Schwartz[10] in its ability to agglutinate trypsin-treated cells. However, at least one major difference was the failure of agglutination reversal to occur in the patient's serum. In order to exclude the possibility that the patient's serum lacked reverser activity, the following test was performed. Trypsin-treated cells were incubated for an hour with the patient's serum. These agglutinated cells were then lightly centrifuged, and the supernatant serum was removed. Five volumes of normal, compatible serum, containing known normal reverser, were then added, and the mixture was again incubated for 2 hours. No agglutination reversal resulted from the addition of the normal serum.

Conversely, the presence of normal reverser activity in the patient's serum was demonstrated in the following way. Agglutinin-free reverser was obtained by the method of Spaet and Ostrom.[8] To destroy agglutinin, normal serum was heated to 63° C. for one-half hour and the patient's serum was heated to the same temperature for one hour. Washed, agglutinated cells were prepared by adding trypsin-treated erythrocytes to normal compatible serum and centrifuging immediately. This serum was promptly removed, and the agglutinate was washed three times in saline solution. Such agglutinated cells do not undergo agglutination reversal spontaneously. Both the patient's and the normal heated sera caused agglutination reversal in 5 minutes when added to washed agglutinated cells. It was concluded that the patient's serum did not lack reverser activity.

Although the physiologic reverser activity of normal sera failed to affect cells agglutinated by the patient's serum, such cells were readily dispersed by NaCN. It had previously been demonstrated by Spaet and Ostrom[8] that cyanide could replace physiologic reverser activity by causing elution of the normal autoagglutinin from the cell surface. The addition of 0.05 ml. of 0.1 per cent NaCN solution to trypsin- or papain-treated cells which had been agglutinated by the patient's serum resulted in rapid dispersal of the cells. Cyanide was active against cells agglutinated by all dilutions of the patient's serum, and the agglutination reversal was permanent, no further agglutination occurring following additional centrifugation. The cells which had been dispersed with NaCN were washed in saline solution and exposed to Coombs' serum. This resulted in agglutination, suggesting the presence of adsorbed protein still on the cell surfaces. Trypsin-treated cells which were exposed to normal serum and allowed to undergo agglutination reversal also became Coombs' positive, whether reversal had been produced by serum reversor or cyanide. Hurley and Dacie[11] also demonstrated this following serum reversal of the normal autoagglutinin. The Coombs' titer of "reversed" cells was 1-20 (not a marked degree of sensitization) in all situations using patient's or normal serum.

To demonstrate that the cyanide effect was specific for the normal trypsin agglutinin and the patient's agglutinin, NaCN was added to other systems. The following types of agglutinated cells could not be dispersed by NaCN:

Group A cells agglutinated by commercial anti-A typing serum, the sensitized cells from a patient with acquired hemolytic anemia agglutinated by Coombs' reagent, trypsin- or papain-treated cells agglutinated by commercial anti-Rh serum, and papain-treated cells agglutinated by the patient's autoagglutinin which had been reversed with NaCN and then reagglutinated with Coombs' sera.

Agglutination reversal of cells clumped by the patient's serum was also accomplished by adding trypsin to these cells. The method was that of Hoyt and Zwicker.[6] To 0.05 ml. of a 2 per cent suspension of trypsinized erythrocytes was added an equal volume of a 1-4 dilution in saline solution of the patient's serum. The mixture was incubated for an hour at 37° C. and then centrifuged. The supernatant was removed and to the agglutinated button of cells were added varying amounts of 1 per cent trypsin made up in a phosphate buffer diluent at a pH of 7.1. To the control tube saline solution was added instead of trypsin. The time required for reversal of agglutination to occur was determined in relation to the quantity of 1 per cent trypsin. After reversal the Coombs' test was performed on the washed reversed cells.

Hoyt and Zwicker[6] reported that the agglutination by normal autoagglutinin could be dispersed by an excess of 1 per cent trypsin. These findings were confirmed. In less than 5 minutes, the addition of excess trypsin reversed the agglutination of trypsin-treated cells by normal serum. Cells agglutinated by the patient's serum were dispersed less rapidly. The rate of reversal was measured by noting the degree of agglutination remaining after definite time intervals. This rate was dependent upon the quantity of trypsin employed with a constant amount of serum. Agglutination-reversal began within 20 minutes and was complete within an hour (Table III). When a great excess of trypsin was used, hemolysis occurred; when less trypsin was used, the washed, dispersed cells were Coombs' positive. A possible explanation of these data is that trypsin induced agglutination reversal by excessive digestion of the erythrocyte surface, thus rendering it incapable of participating in the agglutination reaction.

TABLE III. REVERSAL TIME OF AGGLUTINATED TRYPSINIZED CELLS IN EXCESS TRYPSIN

1% TRYPSIN ADDED (ML.)	TIME OF BEGINNING REVERSAL	TIME OF COMPLETE REVERSAL
Patient's Serum		
0	No reversal	No reversal
0.05	40 minutes	60 minutes
0.1	40 minutes	60 minutes
0.2	40 minutes	60 minutes
0.35	20 minutes	50 minutes
0.5	20 minutes	50 minutes
Normal Serum		
0	No reversal	No reversal
0.05	—	5 minutes

V. *Trypsin Inhibitor.*—

In order to clarify the relation of normal trypsin inhibitor found in all sera to our patient's agglutinin, further studies were undertaken based on the methods of Hoyt and Zwicker.[6] The antitryptic activity of the patient's

serum was compared to that of a normal control by measuring inhibition of tryptic digestion of the gelatine on an x-ray film. Both sera had equal ability to inhibit the activity of a standardized trypsin solution (Table IV). In addition, the patient's antitrypsin was neutralized by an excess of trypsin, and the serum tested for its agglutinating ability. The agglutinin was not inhibited by trypsin. These data indicate that the abnormal agglutinin was not a result of antitrypsin activity.

TABLE IV. TRYPSIN INHIBITOR OF NORMAL COMPARED TO PATIENT'S SERUM*

	DILUTION OF SERUM								
	1-1	1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256
Normal serum with trypsin	0	0	0	2	2	3	3	4	4
Patient's serum with trypsin	0	0	0	2	2	3	3	4	4

0 = no digestion
2 = 50 per cent digestion
3 = 75 per cent digestion
4 = complete digestion

*0.05 ml. of serum dilution was mixed with 0.05 ml. of 1 per cent trypsin diluted 1-8 in saline solution. Mixture was incubated at 20° C. for one-half hour, following which 0.05 ml. was added to the x-ray film. Digestion of films proceeded for one-half hour at 37° C.

DISCUSSION

The clinical and laboratory findings in this patient indicate the presence of an unusual form of idiopathic acquired hemolytic anemia. Evidence favoring the diagnosis of a hemolytic process included recurring episodes of anemia, icterus, reticulocytosis, erythroid hyperplasia of the bone marrow, and the finding of a shortened erythrocyte survival time. The unusual features were the presence of hemoglobinuria indicating acute intravascular rather than intracellular hemolysis, lack of hemolysis between episodes despite persistence of a potent autoagglutinin, and the presence of an antibody which affected trypsin-treated erythrocytes without producing a positive Coombs' reaction. This is in contrast to the usual clinical picture of idiopathic acquired hemolytic anemia in which there is a chronic and persistent hemolytic process with a positive Coombs' test.

The occurrence of sudden severe hemoglobinuria followed by acute renal failure as the presenting symptoms of a recurrent attack of hemolysis is most uncommon in "idiopathic" acquired hemolytic anemia. Because of these unusual features idiopathic acquired hemolytic anemia must be considered in the differential diagnosis of other types of hemoglobinuria which include paroxysmal cold hemoglobinuria, paroxysmal nocturnal hemoglobinuria, favism, march hemoglobinuria, blackwater fever, and certain drug sensitivities. Each of these other conditions has a characteristic clinical and laboratory picture which differs strikingly from that of our patient.

Although the antibody pattern of our patient's serum differed from that most frequently described among patients with "immune" hemolytic anemia, it appears similar to those reported by Dausset,[2] by Dacie and Cutbush,[3] and by Dacie.[4] The cases of Dacie and Cutbush differed in that they were all Coombs' positive, although the antibodies of the patients' sera and of the cell eluates exhibited preference for trypsin-treated erythrocytes. Dacie's

case, in which the Coombs' test became negative during remission despite persistence of serum activity with trypsinized cells, is also different in pattern, for our patient, to date, has not exhibited a positive antiglobulin reaction. Whether these differences are of minor character cannot be ascertained as yet. The significance of all these reported cases is that they (a) underline the diversity of antibody patterns which may occur and (b) emphasize that a full battery of diagnostic procedures is necessary for accurate classification of the disease process.

The agglutinin found in the present case is in many respects similar to that found in normal subjects by Rosenthal and Schwartz[10] and Spaet and Ostrom.[8] The normal panagglutinin reacts with trypsin-treated erythrocytes and has been reported to be present to a titer of 1-8 to 1-128 in all persons studied. A similar agglutinin was described by Hurley and Dacie[11] in a single patient, which attained a titer of 1-1,000, and was associated with a hemolysin. In contrast to our own case, these agglutination reactions were readily reversed after a suitable period of incubation in homologous serum. The similarities and differences between the normal autoagglutinin and our present agglutinin are summarized in Table V. For comparison, the properties of the usual agglutinin of acquired hemolytic anemia are included. Whether the differences are due merely to quantitative variations in agglutinin titer, or whether our patient's agglutinin is actually an augmented normal panagglutinin is open to question.

Using an in vitro technique, Bonnin and Schwartz[12] have shown that the normal serum autoagglutinin is capable of stimulating erythrophagocytosis. These same investigators showed that the serum of the patient of Hurley and Dacie had a more marked ability to produce this reaction. These data suggest that such agglutinins may be responsible for cell destruction under certain circumstances.

TABLE V. COMPARATIVE PROPERTIES OF AUTOAGGLUTININS

	SERUM OF PATIENT MN	NORMAL AUTOAGGLUTININ	ACQUIRED HEMOLYTIC ANEMIA
Coombs' test	Negative	Negative	Positive
Trypsinized RBC titer	1-1,024	Usually under 1-128	Variable
Thermal range of activity	No change 4° to 37° C.	No change 4° to 37° C.	Usually most active at 37° C.
Agglutination reversal in serum	No	Yes	No
Agglutination reversal by sodium cyanide	Yes	Yes	No
Agglutination of papainized cells	Yes	No	Yes
Temperature to inactivate	65° C.	62° C.	Stable at coagulation point of serum

It seems possible that the agglutinin of our patient was able to produce hemolysis when the cells were sensitized by some common exogenous agent such as a virus or a bacterial metabolite. A mechanism of this type is suggested by the relationship between the urinary infection and the onset of hemolytic symptoms.

SUMMARY

A case of idiopathic acquired hemolytic anemia is reported which was unusual in two respects: (1) the presenting symptom was hemoglobinuria, which was sufficiently severe on one occasion to produce acute renal insufficiency, but which showed prolonged periods of complete clinical remission; (2) the serum showed an agglutinin which was active against trypsinized erythrocytes, although the patient's red blood cells were Coombs' negative.

Detailed studies were undertaken to characterize the patient's agglutinin. In many respects, this agglutinin resembled that found in the serum of normal persons tested against trypsinized cells. However, it differed in the following respects: (1) the titer was considerably higher than that normally encountered; (2) the agglutination was not reversible with incubation in serum; (3) papain-treated cells were susceptible to agglutination.

It is concluded that the trypsin test is a necessary adjunct in the investigation of acquired hemolytic anemia.

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CHROMATOGRAPHIC ESTIMATION OF FOUR DIFFERENT HUMAN HEMOGLOBINS

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NORMAL adult hemoglobin (A), fetal hemoglobin (F), and sickle-cell hemoglobin (B) have been known for some time. Hemoglobin C,[1] D,[2] E,[3] and G[4] have been described more recently. Filter paper electrophoresis and the Tiselius moving boundary method are used most commonly to study closely related hemoglobin proteins. Other methods include study of increased resistance to alkali, differences in solubility, position of tryptophan fine structure band (hemoglobin F), and decreased solubility of the reduced form of hemoglobin B.

In 1953, Boardman and Partridge[5] succeeded in separating two different hemoglobins, sheep fetal and bovine carboxyhemoglobin, using column chromatography with the cation exchange resin Amberlite IRC 50.[6-10] We have attempted to develop this method for use in the separation of human hemoglobins A, B, C, and F. The separation of human hemoglobins A and F by paper electrophoresis has been especially unsatisfactory. Furthermore, column chromatography makes it possible to isolate the various abnormal hemoglobins in a pure state.

MATERIAL AND METHODS

1. *Hemoglobin Solution.*—The erythrocytes of fresh heparinized blood were washed four times with normal saline solution. Hemolysis was then produced by adding 1 volume of distilled water and 0.4 volume of toluene. The solution was filtered above Celite 545 and then dialyzed at 0° C. for two days against distilled water. The solution obtained was adjusted to an approximate 10 per cent concentration by adding distilled water and the hemoglobin conveyed into the carboxy form. Blood samples were used in this study from normal adults, umbilical cord, patients with sickle-cell anemia and sickle-cell trait, patients with homozygous and heterozygous hemoglobin C disease,[11] a child suffering from Cooley's anemia, and an adult with the heterozygous form of this disease.

2. *Chromatographic Technique.*—Columns 20 by 0.9 cm., filled to 13 cm. with Amberlite IRC 50 (XE 64),* were used for the separation of carboxyhemoglobins F, A, B, and C.[5] This exchange resin had been pretreated.[7] The column was equilibrated with about 200 ml. of the first elution buffer. It is necessary to make two changes during the elution of 30 to 60 mg. of carboxyhemoglobin: to switch over to buffers with a higher sodium ion concentration and to increase the flow rate of the effluent. The first elution solvent is a citric acid buffer, pH 6.50, with a citric acid concentration of 10 Gm. $C_6H_8O_7 \cdot H_2O$ per liter and a fixed sodium ion concentration (0.20). Fetal hemoglobin was found to be little absorbed when this buffer solution was used. Two liters of this solution can be prepared by dissolving 20 Gm. $C_6H_8O_7 \cdot H_2O$ in about 1 liter of distilled water and adjusting the pH to 6.50 with 4 N NaOH. The sodium ion concentration is then 0.135 and may be adjusted to 0.20 by adding 7.605 Gm. NaCl. At an effluent volume of about 6 ml., and just before

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the emergence of carboxyhemoglobin F, a buffer with a sodium ion concentration of 0.25 is added above the resin. This buffer is prepared as outlined previously except that 13.455 Gm. NaCl is added. For the elution of carboxyhemoglobin C, a final change to a buffer with sodium ion concentration 0.30 (addition of 19.305 Gm. NaCl) is made after the emergence of normal adult hemoglobin (effluent volume 20 to 22 ml.).

The effluent fractions are collected during the first hours at a maximum rate of 0.6 ml. per 30 minutes. The volume of the fractions is increased to 3.0 ml. per 30 minutes after the elution of carboxyhemoglobin F, at about 11 ml. A slow flow rate is necessary

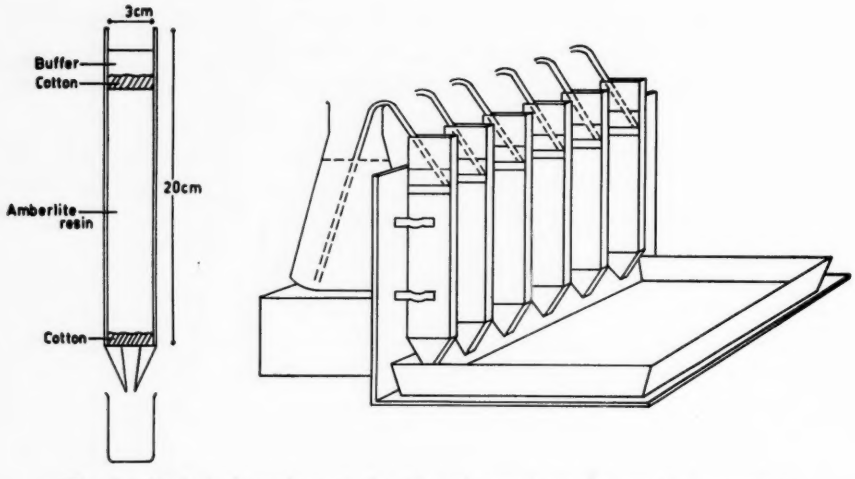

Fig. 1.—Technical equipment for the chromatography on flat cuvettes.

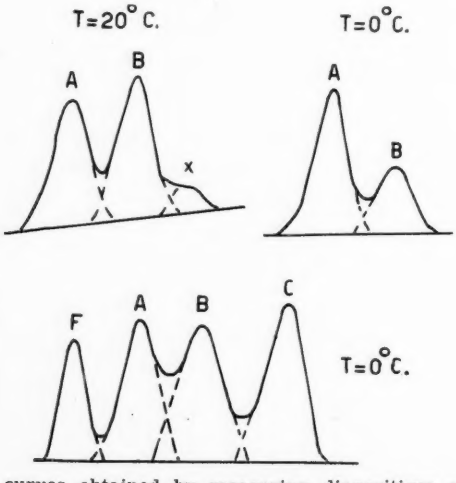

Fig. 2.—Optical density curves obtained by measuring diapositives of some cuvettes by the method of Grassmann and Hannig.[12]

for the separation of carboxyhemoglobins F and A, while increased speed of elution has no perceptible influence on the separation of the other components. Increased flow rate thus shortens the necessary time considerably. All the fractions are finally diluted to 4.0 ml. and the extinction is measured at 5,700 A.U. with a Beckman spectrophotometer. Because the whole experiment requires about 24 hours it is important to perform the experiment at a temperature of 0° C. to avoid the formation of methemoglobin.

More recently, we have devised a more simple and rapid method in which no effluent fractions are collected and the various hemoglobins are visualized on the column as separate bands. A flat cuvette measuring 20 by 3 by 0.5 cm. inside is filled to a height of 15 cm. with the same resin described before. The cuvette is clear Lucite and is occluded at each end with cotton as shown in Fig. 1.

One milliliter of an approximately 2 per cent concentration of the material to be chromatographed is added to the surface of the resin. The buffer used is sodium citrate-citric acid with a sodium ion concentration of 0.15 and pH of about 6. One liter of stock solution is prepared by dissolving 147.0 Gm. sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$) in about 500 ml. of distilled water and adjusting the pH to 6 by means of a concentrated citric acid solution. This solution is diluted 10 times and may be siphoned from a supply bottle to pass through the cuvette at a maximum rate of 20 ml. per hour (Fig. 1). A good separation of hemoglobins is visualized in 10 hours after about 200 ml. of buffer has passed through the cuvette.

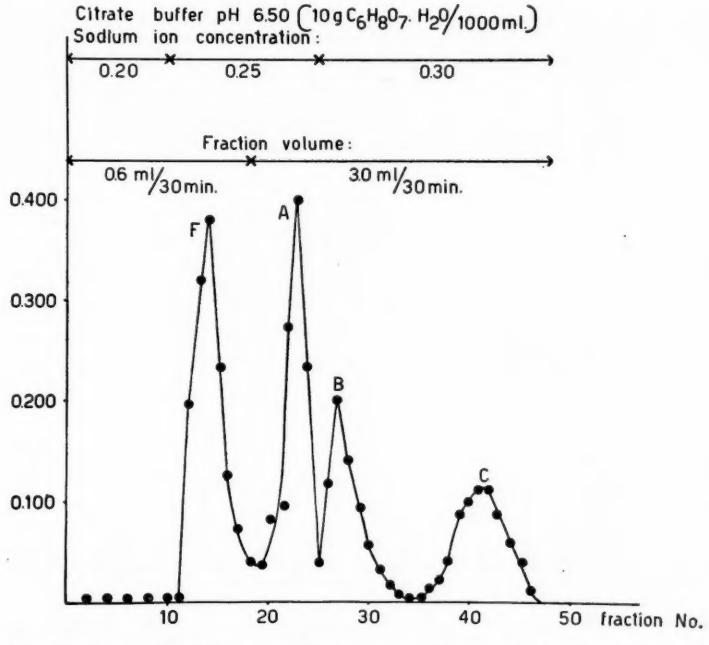

Fig. 3.—Elution curve of different hemoglobins on a column of 13 by 0.9 cm. Amberlite IRC 50 (XE 64).

Percentages of the various hemoglobins may be estimated by measuring a diapositive of the cuvette.[12] Fig. 2 presents an example of an estimate of the amounts of carboxyhemoglobin F and A in a mixture of these two hemoglobins.

Methemoglobin becomes visible as a third colored band at 20° C. This chromatographic method simultaneously may be carried out with several cuvettes in a refrigerator.

3. *Other Methods.*—These two methods were compared with the Tiselius and the alkali denaturation methods[13] in determining the quantity in per cent of fetal hemoglobin. The hemoglobin, rather than carboxyhemoglobin, was subjected to electrophoresis in a Tiselius apparatus.* A 1 Gm. per cent concentration solution was used. A 0.02 M phosphate buffer pH 6.75 was used to which 0.03 M sodium chloride was added. The determination was carried out for 6 hours at 5.5 MA. and 120 V.

*Perkin Elmer Company.

RESULTS

An example of the separation of four different human hemoglobins is presented in Fig. 3. Carboxyhemoglobin F moves fastest. The other three components are eluted in the order of A, B, and C. The separation of the four pigments is almost complete and allows a quantitative determination. A total recovery of 85 per cent was made out of 48.5 mg. of hemoglobin chromatographed. The recovery for each of the four separate hemoglobins was 97 per cent for F, 81 per cent for A, 90.5 per cent for B, and 72.5 per cent for C, at a temperature of 10° C. At 0° C. the recoveries were F, 97 per cent; A, 99 per cent; B, 94 per cent; and C, 106 per cent. These findings emphasize the desirability of carrying out the analysis with freshly prepared blood samples at low temperatures.

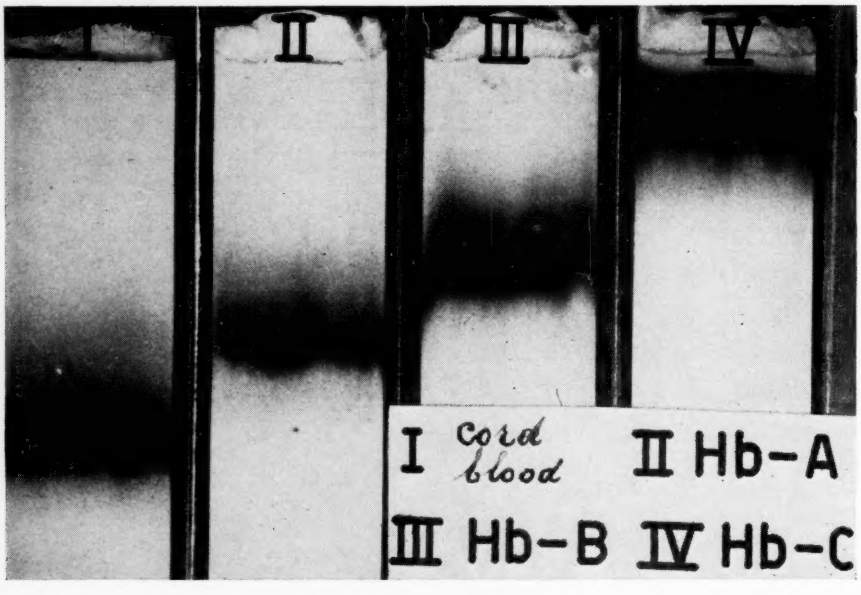

Fig. 4.—Diagram of different hemoglobins on flat cuvettes after passing of a constant amount of the effluent.

Fig. 4 presents pictures of the directly visualized results of chromatographing four hemoglobins separately by the simplified cuvette method. Again, F moves fastest (Sample I). The other three hemoglobins follow in the order of A, B, and C. Fig. 5 demonstrates that the four different hemoglobins can also be separated on one column. The mean rates of displacement of the four examined hemoglobins appear to be fairly constant. If the rate for A is taken as 1.00, the rate for F is 1.33 (range 1.23 to 1.43); for B, 0.72 (0.67 to 0.79); and for C, 0.34 (0.30 to 0.40). A band may be identified by these ratios or by adding known hemoglobins. Fig. 2 presents the curves obtained by measuring the optical density on the diapositive of the cuvette for a mixture of A, B, and for a mixture of the four hemoglobins studied. In the latter mixture there is slight overlap of A and B.

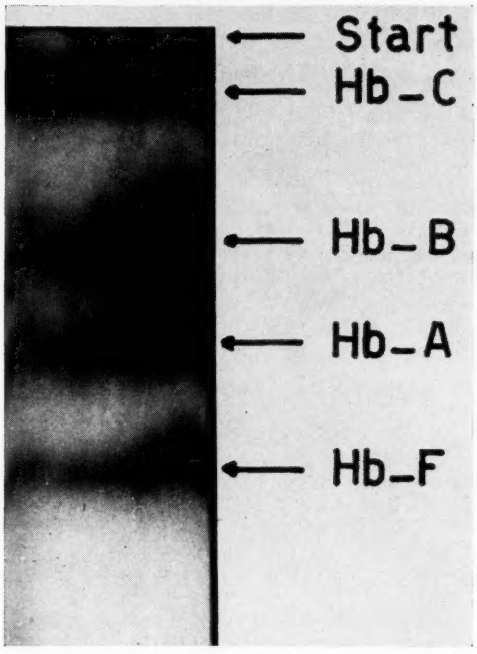

Fig. 5.—The separation of four different human hemoglobins on one flat cuvette

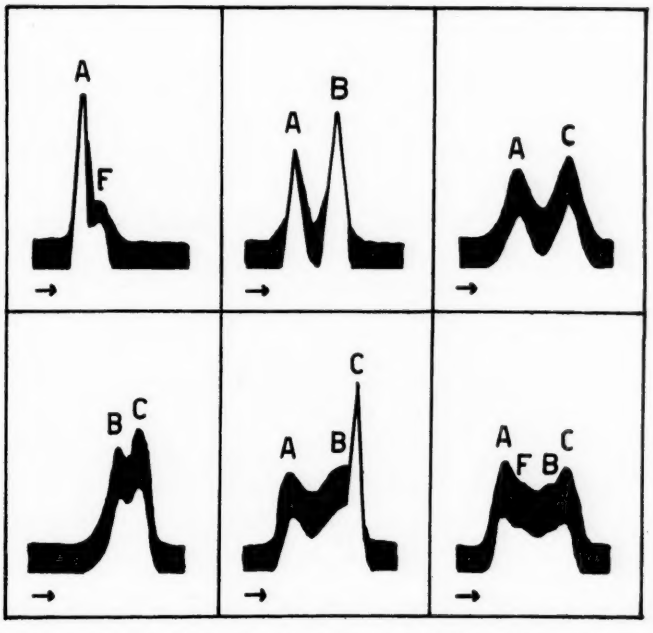

Fig. 6.—Electrophoretic diagrams of mixtures of different human hemoglobins.

Fig. 6 presents some results of Tiselius electrophoresis of mixtures of various hemoglobins for comparison. It appears that almost complete separation into two components may be obtained with a number of combinations of hemoglobins (A + B, A + C). The separation is less distinct with other mixtures (A +F, B + C), particularly if one of the components is present in a small concentration. Mixtures with three or four hemoglobins show a fairly incomplete separation by the Tiselius apparatus in contrast with the separation obtained with the chromatographic techniques (Figs. 2, 3, and 5).

TABLE I. THE ESTIMATION OF FETAL AND ADULT HEMOGLOBIN IN MIXTURES OF COHb-A AND COHb-F ESTIMATED BY THE TWO CHROMATOGRAPHIC METHODS AT 0° C.

	CALCULATED		ELUTION METHOD			CUVETTE METHOD	
SAMPLE	COHb-F	COHb-A	COHb-F	COHb-A	TOTAL RECOVERY (F + A)	COHb-F %	COHb-A %
1	83.5*	16.5	83; 79†	14; 15†	97; 94†	91.5; 91.5†	8.5; 8.5†
2	72.5	27.5	71; 70	23.5; 25.5	94.5; 95.5	79; 77.5	21; 22.5
3	59	41	58; 50.5	36; 39	94; 89.5	61; 61	39; 39
4	42.5	57.5	43.5; 42.5	46.5; 57.5	90; 100	49.5; 47.5	50.5; 52.5
5	24	76	24.5; 24.5	62.5;	87;	22; 19.5	78; 80.5
6	12	88	11; 15.5	76; 75.5	87; 91	12.5; 12.5	87.5; 87.5

*Estimated by the alkali denaturation method of Brinkman and Jonxis[13]; the percentages of Hb-F in the other five samples were obtained by dilution with normal adult hemoglobin.
†Duplicate estimations.

Table I presents a comparison of quantitative results obtained by these two chromatographic techniques in the estimation of fetal and adult hemoglobin from a number of artificial mixtures of these two hemoglobins. These results are compared with the calculated values. The quantities of F estimated by the chromatographic method correspond closely to the calculated

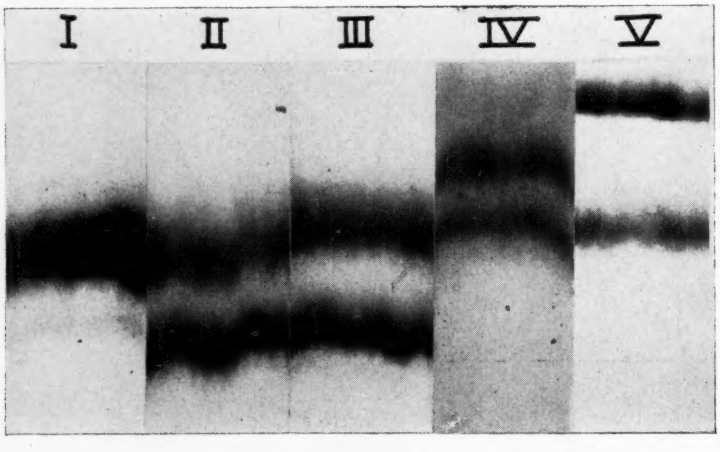

Fig. 7.—Application of the cuvette method in blood samples of some patients with abnormal hemoglobins. *I*, Cooley trait; *II*, Cooley trait after addition of Hb-F; *III*, Cooley's anemia; *IV*, sickle-cell trait; *V*, heterozygous Hb-C disease.

values. The duplicate estimations show but slight differences, but for a single exception. Recovery of adult hemoglobin and total hemoglobin is less complete, especially when adult hemoglobin is present in relatively large quanti-

ties. Carboxyhemoglobin A may be more susceptible to conversion to methemoglobin than fetal hemoglobin. The range of error in the determination of adult hemoglobin is about 10 per cent.

The quantities of carboxyhemoglobin A found in samples rich in this hemoglobin by the cuvette method are more closely in accord with the calculated values. Estimates of adult hemoglobin in samples containing a low concentration of this hemoglobin show values erroneously low. Duplicate determinations check closely, however.

Fig. 7 presents some examples of the clinical application of the cuvette method. Sample I is the hemoglobin of a patient with thalassemia minor. Thirteen per cent hemoglobin F was found with the method of alkali denaturation,[13] 9 per cent by electrophoresis, and 10 per cent by the cuvette method. The presence of F in this specimen was more definitely ascertained as shown in Fig. 7,^{II} by adding hemoglobin from umbilical cord. Fig. 7,^{III} shows the chromatogram of the hemoglobin of a son of the same patient. The son presented the clinical picture of Mediterranean anemia. The fetal hemoglobin content of his blood was found to be 43 per cent by the alkali denaturation method. The cuvette method demonstrated 43, 40, and 44 per cent in triplicate determinations. Fig. 7,^{IV} shows the chromatographic separation of A and B from the blood of a patient with sickle-cell trait. The separation of B is easily visualized. Carboxyhemoglobin A and C were also distinctly visualized (Fig. 7,^{V}) in the blood of a patient suffering from the heterozygous C disease.

DISCUSSION

It is known from electrophoretic experiments that using a buffer of pH 6.5 and ionic strength 0.1, the mobility of hemoglobins increases in the order $A < F < B < C$. It is possible that these differences in charge of the hemoglobin molecule are also of importance in chromatographic separation. The structure of the molecule and length of peptide chains may also be important. These differences might explain the difference in order of electrophoretic mobilities ($A < F < B < C$) as compared to rate of displacement by the chromatographic elution method ($F > A > B > C$).

Carboxyhemoglobin F, which moves rapidly by chromatography, has a smaller number of basic amino acids than A.[14] Carboxyhemoglobin C, however, moves more slowly and has a considerably larger number of lysine residues.[15] Carboxyhemoglobins A and B show little difference in amino acid composition. Determining factors in chromatographic separation of the four hemoglobin fractions may be different therefore in amounts of basic amino acids, in isoelectric points, and structural differences.

SUMMARY

Two chromatographic methods are described for use in the quantitative separation of human hemoglobins A, B, C, and F. The first method utilizes column chromatography with elution. The second method is simpler and more

rapid and allows direct visualization of fractions without elution. Results with these two methods of separating human hemoglobin fractions compare favorably with existing methods of distinguishing hemoglobins.

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FACTORS AFFECTING THE RATE OF REMOVAL OF GELATIN-STABILIZED RADIOGOLD COLLOID FROM THE BLOOD

I. The Retardation of the Radiogold Disappearance Rate by Gelatin

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THE rate of removal of colloidal particles from the circulation has been used as a criterion of the functional activity of the reticuloendothelial system (RES).[3] In these studies it has been assumed that all the particles were presented in a form that allowed the maximal rate of phagocytosis. In attempting to use gelatin-stabilized radioactive colloidal gold to investigate RES activity, it was found that the concentration of gelatin markedly affected the disappearance rate of the radiogold from the circulation. A survey of the literature indicated that with the exception of India ink,[10] there was little, if any, information on the effects of colloidal stabilizing agents on the rate of removal of particulate material from the blood. It was decided, therefore, to study the role of gelatin in the disappearance rate of colloidal radioactive gold from the circulation.

Increasing concentrations of gelatin caused a corresponding reduction in the disappearance rate of the gold. The mechanism of this action was then investigated and was found to be related to the circulating level of alpha and beta globulins rather than to a nonspecific protein effect. This may indicate a new factor of some importance in the clearance of particulate material from the circulation by the RES.

MATERIALS AND METHODS

The particulate material used in this investigation was a commercially prepared gelatin-stabilized radiogold colloid.* The radiogold had an activity of approximately 15 mc. per milliliter on delivery and was diluted with sterile demineralized water in the proportion of 1 part of radiogold to 50 parts of water. In order to prevent aggregation of the gold particles, the syringes, needles, and siliconized vials used in handling the diluted radiogold were sterilized. The radiogold was stored in a refrigerator.

The gelatin used was Knox Special Solution,† salt-free and containing 60 mg. of gelatin per milliliter. A small volume of gelatin, usually 3 to 5 ml., was removed from the stock bottle under sterile conditions and placed in a sterile vial. The vial was placed in a constant temperature water bath at 40° C. for at least 15 minutes before injection. The lyophilized rat plasma was prepared in this laboratory from rats of the same strain as those used in the experiment. The human plasma fractions were obtained through the courtesy of the American Red Cross. The amounts of additional gelatin, rat plasma, and human plasma fractions which were injected had been determined on the basis of milligrams per 100 grams of body weight.

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*Abbott Aurcoloid.

†Obtained from Charles B. Knox Gelatin Products, Inc., Camden, N. J.

Adult male rats of the Wistar strain, weighing between 250 and 300 grams, were anesthetized by an intraperitoneal injection of sodium Cyclopal.* A transverse skin incision was made in the neck to expose both external jugular veins. Injections were made into the right external jugular vein and withdrawals made from the left. Gelatin, plasma, and plasma fractions were injected 15 minutes before the radiogold colloid to minimize the effects due to plasma volume changes.

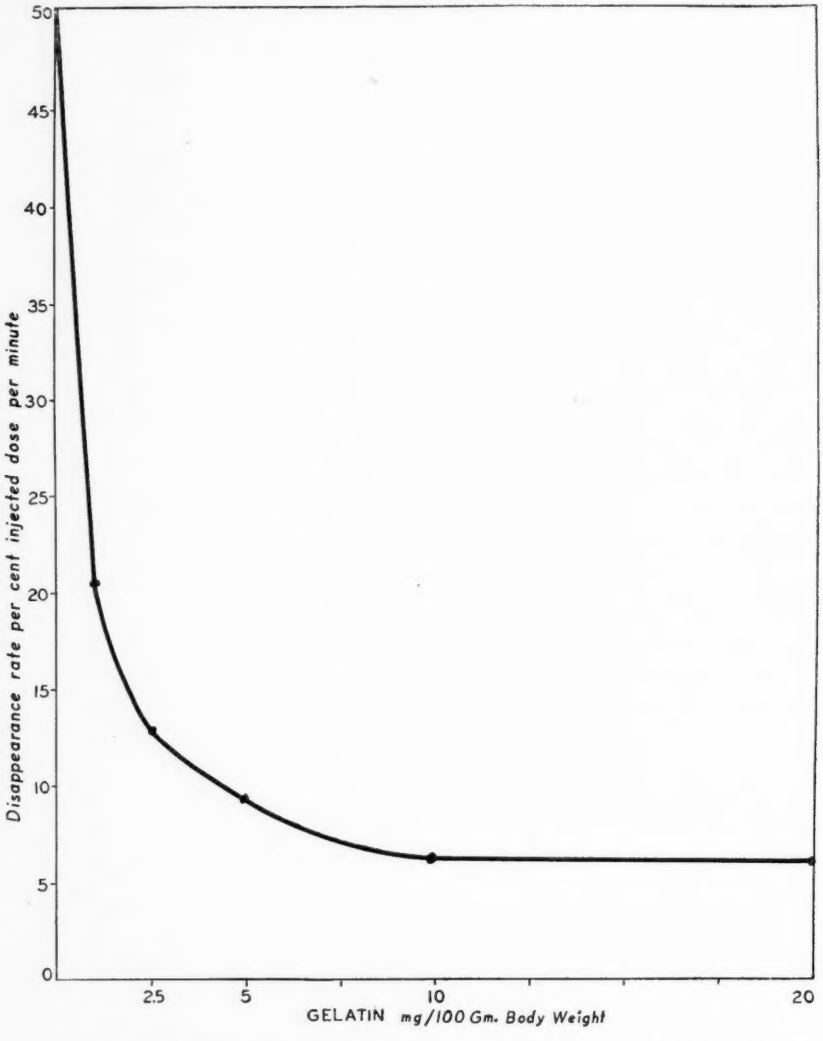

Fig. 1.—The effect of increasing amounts of gelatin on the per cent of injected radiogold cleared from the circulation per minute.

Blood samples of approximately 0.15 ml. were withdrawn in heparinized syringes at intervals of 3 to 5 minutes with a minimum of four samples per rat. A measured volume (0.05 ml.) of blood from each sample was pipetted onto steel planchets and 0.25 ml. of a 10 per cent aqueous solution of liquid detergent (7X)† added. After drying, the planchets were counted under a Geiger-Müller counter.

*Courtesy of The Upjohn Company, Kalamazoo, Mich.
†Obtained from the Linbro Chemical Company, New Haven, Conn.

The total radioactivity of the liver and spleen was determined at intervals after the injection of radiogold by the following procedure. One minute before the stated time interval, both external jugular veins were severed and 20 ml. of saline solution was injected into the abdominal aorta. The organs were removed, weighed, and digested with 5 ml. of 1 N NaOH per gram of tissue in a boiling water bath. The digested organs were diluted to volume with distilled water. Aliquots, in duplicate, were placed in glass vials, counted in a well-type scintillation counter and compared with standards prepared by diluting the radiogold colloid to volume with distilled water.

RESULTS

The total amount of gold injected varied between 0.006 and 0.024 mg. as calculated on the basis of the stated concentration of the original colloid gold preparation. There was no detectable difference in the disappearance rates between these minimum and maximum amounts of gold.

The rate of removal of radiogold colloid from the blood was observed to follow an exponential function. Plotting the blood radioactivity in counts per minute against the time in minutes on semilogarithmic paper gave a straight line, as has been found previously.[1] The time taken for removal of 50 per cent of the circulating radiogold, designated by $T\frac{1}{2}$, was read directly from the graph.

Retardation of the Disappearance Rate by Gelatin.—There was a marked decrease in the rate of removal of circulating radiogold following the injection of small amounts of gelatin (Table I). The disappearance rate of radiogold

TABLE I. EFFECT OF GELATIN AND GELATIN-PLASMA PROTEIN ON THE RATE OF REMOVAL OF RADIOGOLD FROM THE CIRCULATION

TREATMENT	AMOUNT INJECTED MG./100 GM. BODY WEIGHT		NO. OF ANIMALS	$T\frac{1}{2}$ IN MINUTES MEAN ± S.E.
	PLASMA PROTEIN	GELATIN		
Saline solution*	–	–	6	1.0 ± 0.08
Saline solution†	–	–	6	1.0 ± 0.09
Gelatin	–	1	5	2.9 ± 0.16
Gelatin	–	2.5	6	5.0 ± 0.15
Gelatin	–	5	8	7.3 ± 0.28
Gelatin	–	10	6	10.5 ± 0.33
Gelatin	–	20	5	11.3 ± 0.33
Human serum albumin	20	–	4	1.2 ± 0.10
Human serum albumin	40	–	4	1.2 ± 0.15
Human serum albumin + gelatin	20	2.5	4	5.1 ± 0.15
Human serum albumin + gelatin	40	2.5	4	5.0 ± 0.11
Human serum albumin + gelatin	20	5	4	7.3 ± 0.11
Human serum albumin + gelatin	40	5	4	7.4 ± 0.32
Homologous rat plasma	40	–	4	1.2 ± 0.15
Homologous rat plasma + gelatin	40	1	5	8.3 ± 0.24
Homologous rat plasma + gelatin	40	2.5	5	15.3 ± 0.33
Homologous rat plasma + gelatin	40	5	6	19.2 ± 0.58
Human plasma fraction II	5	–	4	1.1 ± 0.11
Human plasma fraction II + gelatin	5	2.5	5	7.5 ± 0.37
Human plasma fraction II + gelatin	5	5	5	8.2 ± 0.34
Human plasma fraction IV-4	5	–	4	1.2 ± 0.10
Human plasma fraction IV + gelatin	5	2.5	5	8.3 ± 0.24
Human plasma fraction IV + gelatin	5	5	5	11.8 ± 0.54
Human plasma fraction IV	10	–	4	1.2 ± 0.16
Human plasma fraction IV + gelatin	10	2.5	5	12.9 ± 0.40
Human plasma fraction IV + gelatin	10	5	5	18.7 ± 0.65

*Equivalent to minimum gelatin volume.
†Equivalent to maximum gelatin volume.

alone was 50 per cent of the injected dose per minute. One milligram of gelatin per 100 grams of body weight reduced this rate to 20 per cent of the injected dose per minute (Fig. 1). There was no significant difference between the disappearance rates of radiogold following pretreatment with 20 mg. of gelatin as compared with 10 mg. ($P > 0.05$). Hematuria was produced one hour after the injection of 40 mg. of gelatin per 100 grams of body weight, although it was never apparent when 20 mg. or less was injected.

Enhancement of the Gelatin Effect by Plasma Proteins.—If the rate of disappearance of radiogold was dependent primarily on the amount of nonspecific protein in the blood, rather than on a foreign protein reaction, an increased plasma protein concentration might produce an effect similar to gelatin.

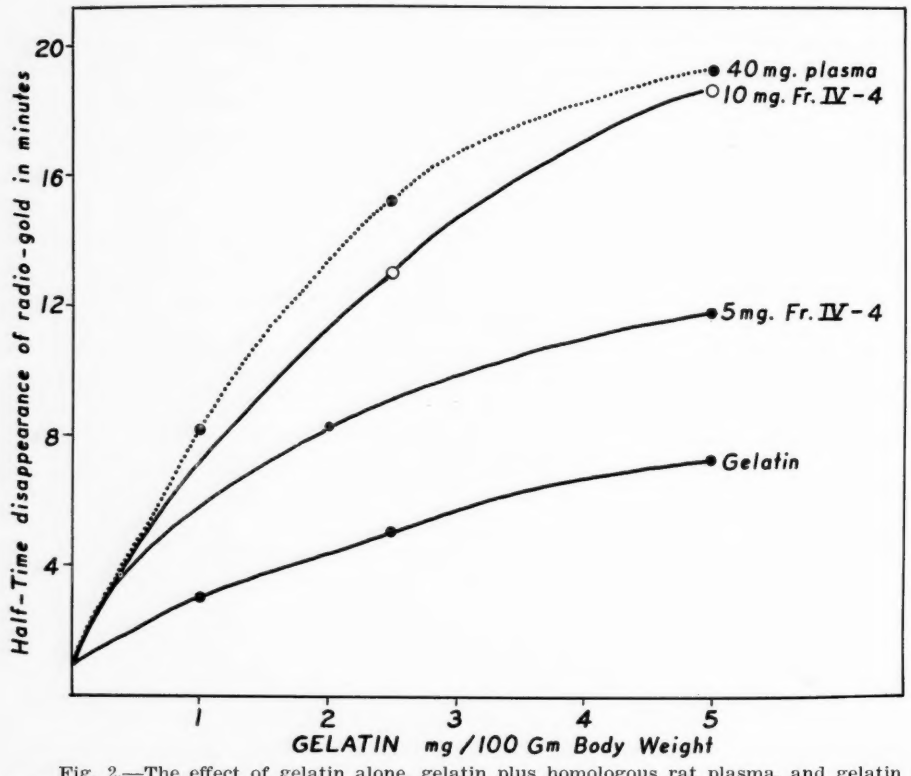

Fig. 2.—The effect of gelatin alone, gelatin plus homologous rat plasma, and gelatin plus human plasma fraction IV-4 on the time taken to remove 50 per cent of the subsequently injected radiogold colloid from the circulation. The amounts of gelatin and plasma protein were calculated on the basis of mg. per 100 Gm. of body weight.

Homologous rat plasma injected alone failed to influence the rate of removal of radiogold, but when combined with gelatin it had a marked effect. The time taken to remove 50 per cent of the injected radiogold was now approximately 2.7 times longer than with the gelatin alone (Fig. 2). Human plasma fraction IV-4, when injected in 5 and 10 mg. quantities, did not decrease the rate of radiogold removal. In combination with gelatin, however, the disappearance rate of radiogold was significantly slower than could be attributed to the gelatin. The differences between the 50 per cent disappearance times for 40 mg. of plasma and 10 mg. of IV-4 fraction are not highly significant ($P > 0.01$).

The intravenous injection of 40 mg. of serum albumin per 100 grams of body weight did not retard the usual rapid disappearance of radiogold from the circulation (Table I). When serum albumin was combined with gelatin, the decrease in the rate of uptake was no different from that obtained with the gelatin alone. Human plasma fraction II, when injected alone and in combination with gelatin, produced a peculiar effect. The blood samples were viscous, the veins were partially collapsed, and the general impression was one of circulatory failure. There was no clear evidence that this fraction enhanced the gelatin effect but rather suggested a relative increase in the concentration of the radiogold colloid in the blood.

Organ Analysis.—Since gelatin might exert its effect by altering the usual sites of ingestion of the radiogold colloid, the total radioactivity of the organs primarily concerned with radiogold removal was measured at various times after injection. The gradually increasing concentration of the radioactivity in the liver with time was inversely proportional to the rate of disappearance of radiogold from the circulation. There was no evidence that the capacity of the liver or spleen to ingest radiogold particles had been altered either by the gelatin or plasma (Table II).

TABLE II. EFFECT OF GELATIN AND GELATIN-PLASMA PROTEIN ON RATE OF UPTAKE OF RADIOGOLD BY LIVER AND SPLEEN

MIN. AFTER Au^{198} INJ.	SALINE		GELATIN* % OF INJECTED Au^{198}		GELATIN + PLASMA†	
	LIVER	SPLEEN	LIVER	SPLEEN	LIVER	SPLEEN
5	87.8 ± 5.1‡	1.5 ± 0.6	56.7 ± 5.1	0.9 ± 0.2	24.3 ± 2.9	0.8 ± 0.3
9	95.3 ± 6.3	1.8 ± 0.8				
13	97.7 ± 3.6	1.5 ± 0.5	71.0 ± 4.9	1.7 ± 0.7	53.2 ± 3.5	1.5 ± 0.6
17			95.5 ± 1.3	0.9 ± 0.3		
25					83.2 ± 4.9	1.8 ± 0.5

*5 mg. per 100 Gm. of body weight.
†5 mg. of gelatin + 20 mg. of homologous rat plasma per 100 Gm. of body weight.
‡Mean ± S.D. with 4 animals for each value.

Although the arterial system was flushed with saline solution, the organs were removed at times when the radioactivity in the blood was relatively high. Therefore, we could not be certain that all of the radioactivity in the liver or spleen actually represented ingested radiogold colloid. If such a bias were introduced, it would tend to minimize the differences between the total radioactivity in the organs of the control and gelatin-plasma treated groups.

DISCUSSION

The finding that increasing amounts of gelatin were associated with the retention of radiogold in the circulation for longer periods of time suggested either a blockade effect of the RE cells by gelatin or the formation of a gelatin-gold-plasma complex in the blood. Although antigenic materials are removed by the RES, there was no evidence from the literature that gelatin, a non-antigenic protein, might be preferentially ingested by the phagocytic cells. The injection of large amounts of an antigen (human serum albumin) had no effect on the rate of removal of the radiogold whereas small amounts of gelatin

had a marked effect. It has been reported[2] that particulate material was removed from the circulation more rapidly following the injection of a foreign protein. While blockade of the RES had been demonstrated[8] after multiple injections of polyvinyl pyrrolidone (PVP), the injection of 60 mg. of PVP per 100 grams of body weight did not decrease the rate of disappearance of subsequently injected radiogold.[6] Furthermore, the disappearance rates of radiogold were similar following the injection of either 10 or 20 mg. of gelatin and more than 95 per cent of the radiogold was removed by the liver regardless of whether or not gelatin had been injected. These findings were not consistent with the theory of RES blockade by gelatin.

The possibility that radiogold was bound by blood proteins was suggested by Simon,[7] who demonstrated by paper electrophoretic separation of plasma from patients receiving radiogold that the radiogold migrated with the alpha and beta globulin fractions. Although this evidence was not proof of binding, it is possible that binding does occur. The finding that plasma and fraction IV-4, but not fraction II or V, enhanced the gelatin effect was consistent with the hypothesis of a gelatin-gold-plasma complex. The rat plasma and fraction IV-4, in the doses injected, contained approximately equal amounts of alpha and beta globulin and the enhancement of the gelatin effect by each was similar. The presence of increased levels of circulating alpha and beta globulins probably allowed more of the radiogold to be bound. The failure of plasma and fraction IV-4, in the absence of gelatin, to retard the clearance of radiogold from the blood suggested that gelatin facilitated the binding of radiogold to the alpha and beta globulins.

The particulate materials most frequently used for investigating the functional activity of the RES are colloidal suspensions of carbon (India ink), thorium dioxide (Thorotrast), gold, and chromium phosphate. Of these, the commercial preparations of India ink* and radiogold† usually contain gelatin which forms a protective coating around the particles and results in a more stable colloidal suspension. The amount of gelatin in these preparations is a trade secret and, therefore, is unknown to the investigator. This uncontrolled factor in the use of gelatin-stabilized colloids and gelatin-free colloids to measure RES function may explain discrepancies in reported experimental data. As for example, Janeso,[5] using a gelatin-stabilized colloid, reported that histamine increased the phagocytic activity of the RES, while Barrow and associates[1] were unable to influence the rate of phagocytosis of gelatin-free colloid by antihistamine drugs. The increased capacity of the RES for a gelatin-stabilized colloid following cortisone treatment, as demonstrated by Timiras,[9] is in contrast to the findings of Heller,[4] who reported that cortisone depressed the ability of the RE cells to phagocytize a gelatin-free colloid. Further studies of this gelatin mechanism are in progress and the preliminary findings suggest that gelatin does not exert its effect at the level of the RE cell.

*Higgins' India Ink.
†Abbott Aurcoloid.

SUMMARY

The rate of disappearance of gelatin-stabilized radiogold colloid from the circulation was dependent primarily on and inversely proportional to its gelatin per 100 grams of body weight decreased the clearance rate of subsequently injected radiogold by 60 per cent. Plasma and plasma fraction IV-4, when injected with gelatin, enhanced the gelatin effect but did not influence the rate of removal of radiogold when injected alone. The possible mechanisms by which gelatin retards the disappearance rate of radiogold are discussed. It is suggested that the decreased rate of clearance of gelatin-stabilized radiogold colloid was due to the formation of a gelatin-gold-plasma complex. The use of blood clearance rates of gelatin-stabilized colloids, as a measure of phagocytic activity, may not be valid.

We wish to acknowledge the helpful suggestions of Dr. Jack Gross and the capable technical assistance of Miss Sheila Stahl.

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CONSTANT MASS DISPLACEMENT BALLISTOCARDIOGRAPHY AND ELECTRICAL IMPEDANCE PLETHYSMOGRAPHY

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HENDERSON[1] was searching for a plethysmograph to record the total mass movement of blood when he rediscovered the recoil curves associated with dynamic cardiac activity. Burger and associates[2] and Rappaport[3] also recently resorted to simple recoil curves of bed displacement to re-evaluate the ballistic forces within the body. Each of these investigators advocated and used light beds and, therefore, variable mass loading of the system from subject to subject.

In this study[4, 5] we have recorded the effect of ballistic activity associated with the heartbeat by the response of a constant heavy mass pendulum bed. Some of these tracings were compared simultaneously with a torso or limb-to-limb plethysmograph and an electrocardiograph. The velocity characteristics in ballistic and plethysmographic systems were recorded electrically. The advantages and disadvantages of these methods for evaluation of mass movement of blood will become apparent from the observations. The results appear to enlighten the controversial concepts already engulfing this important field of cardiodynamics.[6-14]

In a personal conversation with Professor H. C. Burger of Utrecht, he defined the low frequency displacement ballistocardiograph as a "plethysmograph of the total mass of the body during cardiac activity." This implies that the displacement ballistocardiograph is a peripheral resultant measure of mass movements of pulses of blood in the veins as well as in the arteries. The recoil movements so effected are primarily dependent upon cardiac ejection which disturbs differentially, in a polyphasic manner, the equilibrium of the volume pulse in the blood vessels directed toward the head and the feet. The plethysmograph may, in addition, indicate the size of the body segment in relation to gross sequestration or run-off of blood. A plethysmograph, as a rule, is not an index of a continuous or uniform flow of blood. The direct electrical impedance of blood varies with its rate of flow below the Reynolds velocity number 200.[15] This fact influences and limits the use of direct electrical plethysmography to phasic problems. Changes in velocity of flow associated with venous occlusion minimize the value of the long-accepted venous occlusion blood flow techniques by mechanical and electrical means.[16, 17]

In 1903, Professor Einthoven constructed a string galvanometer for recording electrical action currents of the heart from the surface of the body. This

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major historical event diverted interest in the study of cardiac ballistics for thirty-five years. As it may be practical and not just an academic curiosity, we were led to re-evaluate the low-frequency pendulum ballistocardiograph of Henderson by simple experiments in relation to electrical plethysmograph of large body segments.

METHOD

A low-frequency pendulum bed was constructed for this study in the manner of Henderson,[1] except that the 96″ by 31″ platform weighed 108 pounds and each of the vertical hardwood supports weighed 5 pounds. Each of these supports had metal eyes fastened in each end for a free bearing at the bed and upper fulcrums. The pendulum load was varied or made equal from subject to subject by sandbags and other weights to produce a known mass system.[4, 5]

The transducer of the movements of the bed depended upon the linear variations in spatial resistance to a radio frequency current of 140 kilocycles between two electrodes immersed in 0.025 normal potassium chloride. One of these electrodes was fixed for reference.

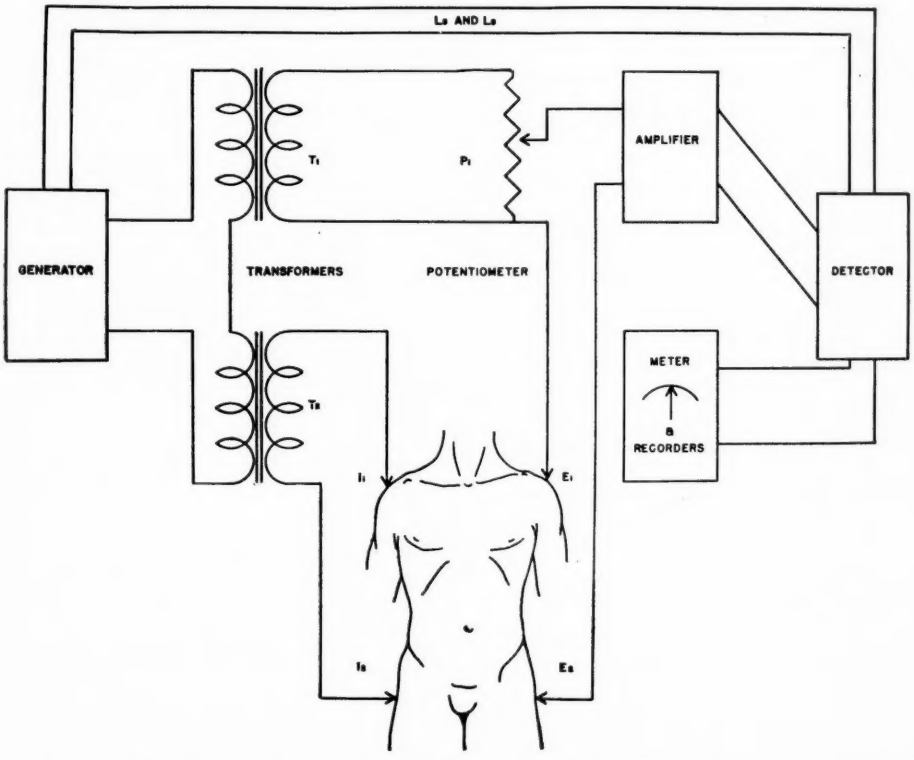


Fig. 1.—Electrical impedance plethysmograph. Schematic circuit of our plethysmograph. It is an electrical device to measure resistive impedance changes associated with changes in volume of blood in various body segments.

The velocity derivative of the displacement measure, exacted from a resistance capacity circuit, was compared simultaneously with the displacement observation (Fig. 4). Any other transducer of displacement giving an accurate definition of the cyclic head-foot movements of the bed over intervals of two seconds or shorter appears adequate.

The natural undamped oscillations of the bed exceeded 2.5 seconds per cycle. The 25 to 30 per cent of critical damping of the natural oscillations of the bed was accomplished

by friction of a steel blade partially embedded in dry, fine sand and mounted with its flat surface parallel with the head-foot movements of the bed. Under these conditions of observation when a breath is held for 2 to 5 seconds, the motion of the bed will thereafter reflect only the foot or headward mass movements of blood with each heart beat.[2, 5] A properly designed oil system of damping is probably preferable but was not available at the time of this study on the heavy bed.

The construction of the electrical impedance plethysmograph was described previously.[28] To orient the reader, a brief description of this method and its principle is again outlined and illustrated schematically in Figs. 1, 2, and 3.

In general, the blood in a given body segment acts as an electrical short circuit resistance to the flow of electrical current through the rest of the segmental tissue. Thus, with

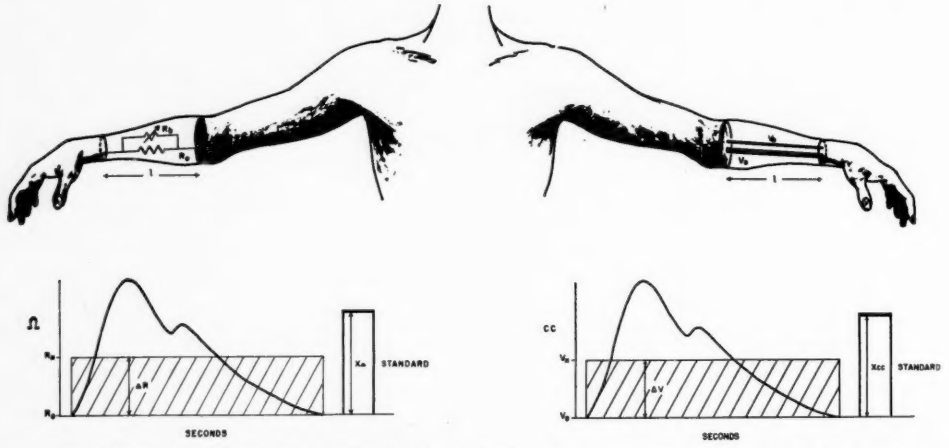


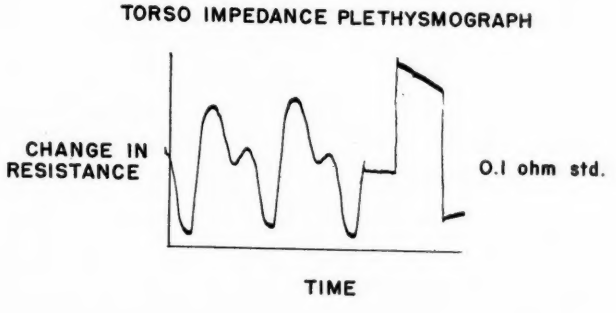
Fig. 2.—The mechanical changes in the volume of an arm segment and its blood contents are represented on the right as parallel tubes. A given pulse, ΔV, its mean height, its comparison standard, and formulation are shown below. The electrical properties in the volume of an arm segment and its blood contents are represented on the left as parallel resistances. A given pulse, ΔR, its mean height, its comparison standard and derivation for volume are shown below:

ELECTRICAL IMPEDANCE METHOD	MECHANICAL METHOD
$\text{Standard} \times \frac{\text{mm height of } \Delta R}{\text{mm height of x ohms}} = R_O - R_N = \Delta R$	$\text{Standard} \times \frac{\text{mm height of } \Delta V}{\text{mm height of } \times \text{ cc}} = \Delta V = V_b$
$\frac{1}{R_N} = \frac{1}{R_O} + \frac{1}{R_b}$	Where V_b = volume of pulse
Where R_b = Resistance of New Blood in Parallel to R_O	$V_b \times$ pulse rate = pulse volume per minute
$R_b = \frac{R_O R_N}{R_O - R_N}$ expressed as $\frac{R_O R_N}{\Delta R}$	Conversion to standard volume allows comparison of segments at different sites
$R = \rho \frac{l}{a}$	
Where ρ = resistance of a 1 cm. cube of blood l = length of segment a = cross sectional area of new blood	
$\rho \frac{l}{a} \cdot \frac{l}{l} = \rho \frac{l^2}{V_b}$	
Where V_b = volume of pulse	
$V_b = \rho \frac{l^2}{R_b}$	

(Figs. 1 and 2 from Nyboer, IRE TRANSACTIONS on Medical Electronics.[28])

each blood pulsation a phasic short circuit and resultant lowering of the resistance of the segment take place (Fig. 2). This resistive impedance change is easily recordable over a wide spectrum of alternating current frequency but has not been practical to record with use of direct current or low-frequency currents.

The impedance plethysmograph technically includes an electrical generator which feeds a radiofrequency current to a network of special transformers interconnected to two or four electrodes on the body segment, a balancing potentiometer, a detector, and recording devices. In practice, the potentiometer value is adjusted manually. It is calibrated in ohms and when properly balanced represents the gross impedance of the biologic or other conductor. The unbalanced changes in impedance are amplified and recorded graphically. These are associated with the blood volume pulse and changes in the volume of the arterial and venous reservoirs produced by posture, muscle movements, or vasomotor activity.[19, 20] The measure is a passive one and discriminates against recording changes due to activity of cells.[18] The low current does not produce heating or a stimulation of irritable tissues. The instrument will therefore evaluate or record all large and small physical changes in terms of the impedance of a conductor, particularly its resistivity when it is altering its blood volume, blood redistribution, or content of ions in any manner. The rheocardiograph[22, 23] used in Europe has a similar function but has usually been constructed with a lower frequency. It is also calibrated in ohms resistivity.



$$\text{PULSATILE VOLUME PER MINUTE} = \frac{\text{VOLUME RESISTIVITY OF BLOOD} \cdot \text{TORSO LENGTH}^2 \cdot 2 \cdot \text{MEAN RESISTANCE OF TORSO PULSE} \cdot \text{PULSE RATE}}{\text{TORSO RESISTANCE}^2} =$$

$$\text{EXAMPLE} = \frac{150 \cdot 42^2 \cdot 2 \cdot 0.05 \cdot 72}{20^2} = 4752$$

Fig. 3.—A sample record and calculation of pulsatile volume displaced per minute in the torso region of a human subject. The volume displaced is about 4,754 c.c. per minute.

Special additional features are often desirable in a plethysmograph besides the ability to detect the volume of the pulse. The velocity or rate of change with which the form of the pulse changes in any direction is recorded as the first electrical derivative of the initial impedance pattern. The acceleration pattern is recordable as the second electrical derivative of the volume pulse. Burch[27] has also recently established these forms from mechanically derived pulse forms; however, Nyboer and his former associates have recorded such derivatives since 1939. They are most useful in following the progression rate of a pulse wave. In general volume, pulses are a nuisance to analyze by planimetry for their mean heights, so systems of integration or summation appear desirable. In this manner it is possible to obtain data representing the rate of sequestration of blood in a segment without venous occlusion. Such occlusive procedures interfere with the existing velocity and distribution of

blood to a segment. A summated measure of mean pulse volume is probably related to the metabolic demand flow of blood, above the level of critical flow to a segment. It does not measure the perfusion flow independent of the volume pulse in a segment.

The method of analysis of the electrical impedance pulse in terms of blood volume is dependent on its parallel resistive relation (R_B) to the segmental resistance (R_O) and its new resistance (R_N) on addition of blood, the volume resistivity of blood (ρ) and the length of the column (l) being measured.[19]

Mr. Roger Shannon depicted our analytical procedure with respect to the arm in Fig. 2. In this manner[4] the pulse volume or the minute pulse volume may be calculated in cubic centimeters as shown in Fig. 3. It is hoped to obtain a similar physical basis and formulation for calculating the mass movement of blood measured by displacement ballistocardiography. In this paper, however, only qualitative and empirical relations are presented and stressed.

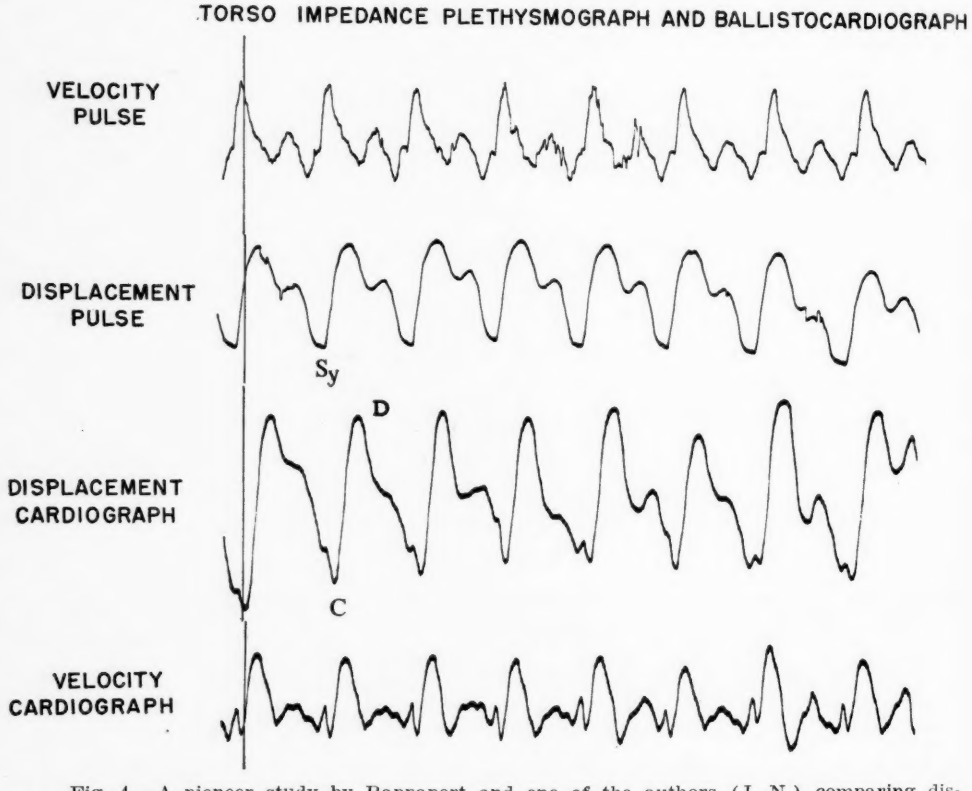


Fig. 4.—A pioneer study by Rappaport and one of the authors (J. N.) comparing displacement and velocity plethysmograms and ballistocardiograms on a lightweight pendulum bed. It is noted that the rise in the volume pulse beginning at (S_y) in the plethysmograph precedes the footward movement (CD) of blood in the displacement cardiogram. The peaks of the chief deflections in velocity pulse occur approximately in the middle of the chief deflection of each displacement curve, respectively. An upward deflection in the displacement pulse is a decrease in segmental resistance which represents an increase in segmental volume of blood between the given electrodes. The paper speed is 25 mm. per second. See Figs. 5 and 6 for nomenclature.

RESULTS

In an early observation (Fig. 4) at the Sanborn Company one of the authors (J. N.) and Rappaport noted the similarity between the forms of simultaneous displacement volume pulses derived from the torso segment and displacement ballistocardiographs recorded from movements of a lightweight Henderson type of bed. It was also noted that the earliest rise in peripheral pulse volume

precedes by 0.04 second the initial downward segment of the ventricular cardiograph ascribed to headward movement of blood. The characteristic direction of events in each system during this time are responsible for the chief differences noted in the form of the corresponding velocity curves of the displacement events (Fig. 4).

The time relation between a bed displacement ballistocardiogram and an electrocardiogram is shown in Fig. 5. The beginning of the ventricular motion initiated by the apex thrust during the isometric contraction of heart is identified in Burger's nomenclature as peak B. It occurs about 0.14 second after the onset of the QRS complex of the simultaneous electrocardiograms.

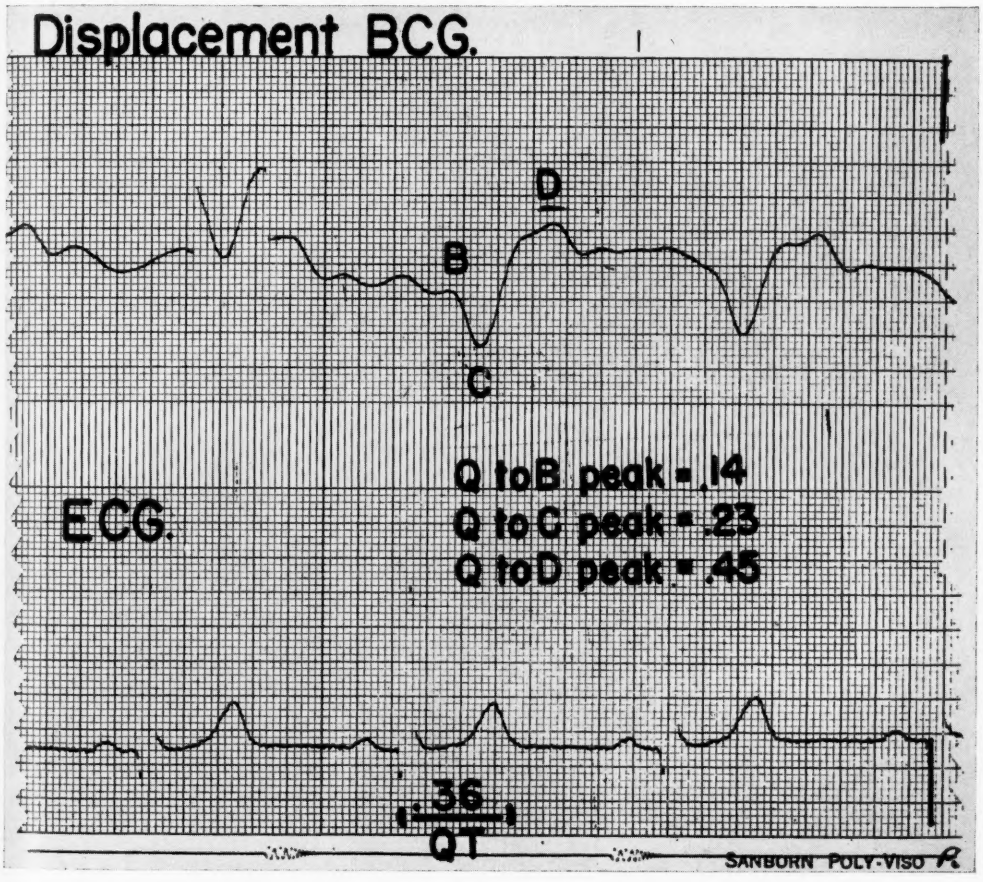


Fig. 5.—Simultaneous electrocardiograms and displacement ballistocardiograms on a heavyweight bed. The earliest change related to ventricular contraction in the ballistocardiogram is represented by peak B. The blood is then said to move in a headward direction (BC) before the bed reverses its movement to produce the deflection (CD) associated with the movement of the blood footward. The time interval between the beginning of QRS in the electrocardiogram and peak *B* is 0.14 second. The paper speed is 50 mm. per second.

This event is followed by a rapid downward displacement component, "BC", which is produced by a footward recoil of the bed associated with initial blood ejection from both ventricles. The inverted peak "C" occurs about 0.23 second after the beginning of QRS in the electrocardiogram. Thereafter, the direction

of the wave alters upwardly with inscription of "CD" while the mass movement of blood is now moving chiefly downward toward the feet. During diastole a prominent notch and plateau, "DEF," occur before restoration of the bed to position B, just prior to ventricular emptying. The characteristics of this diastolic segment of the recoil curve appear to be highly dependent on the venous as well as arterial dynamics but are considered forced movements by Hamilton, Dow, and Remington.[24]

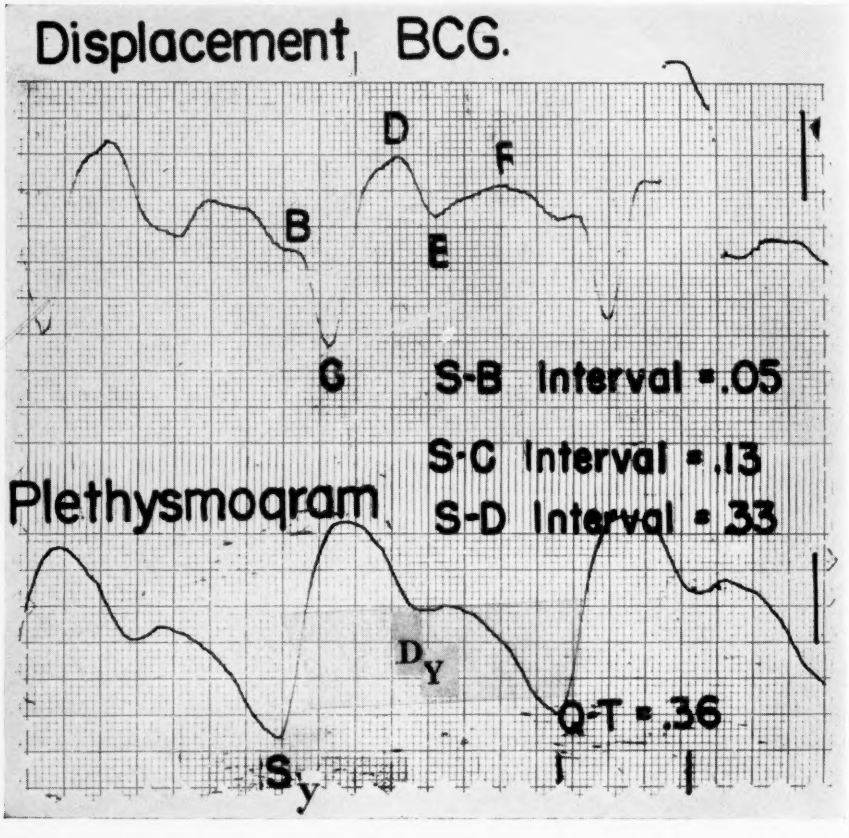


Fig. 6.—A comparison between the displacement ballistocardiograms on a heavyweight bed and the torso electrical impedance plethysmograph. The difference in time of onset of the earliest recorded changes due to movement of blood is 0.05 second as measured from S_y to peak B. The earliest movement of blood is thus detected by the plethysmograph. Inertia is probably a factor in delaying the ballistocardiogram. The duration of systole is nearly equivalent to the Q-T interval in the electrocardiogram. This could be designated as the BE interval in the displacement ballistocardiogram and the S_y-D_y interval in the plethysmograph. An upward deflection in the plethysmograph is a decrease in segmental resistance which represents an increase in segmental volume of blood. Paper speed is 50 mm. per second.

A comparison between this ballistocardiogram and the electrical impedance pulse of the same subject is shown in Fig. 6. It is noted that there is more than 0.12 second difference between the comparable chief rising segment beginning at S_y and C due to outward movements of blood. There is, however, only 0.04 to 0.06 second difference between S_y of the plethysmograph and the onset BC of the ballistocardiograph. This time difference in recorded displace-

ment dynamics in early systole may in part or in entirety be related to the inertia of the bed and mass of the subject since the plethysmographic system probably records instantly the differential mechanical expansion of the peripheral body segments. The form of the two independent displacement tracings related to cardiac ejection is very similar if we compare the general contour of the

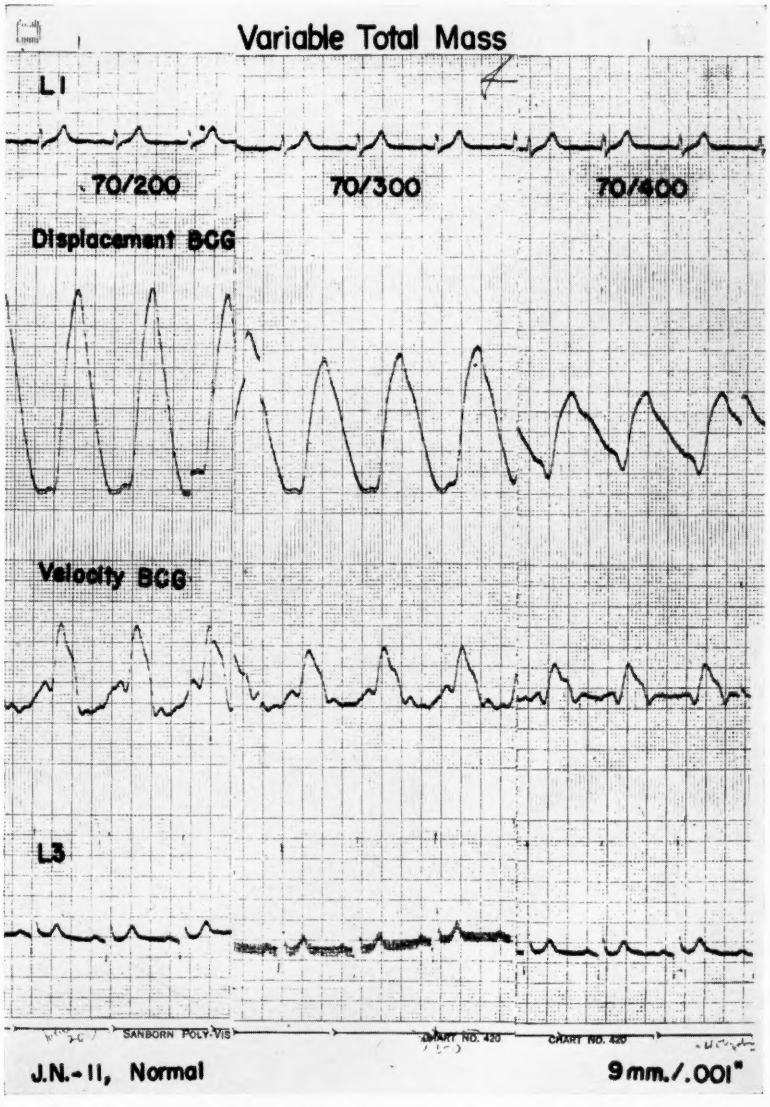


Fig. 7.—The effect of changing the total mass of our bed by 100 pound increments on the form and amplitude of the displacement and velocity ballistocardiograph of a normal 70 pound subject. In an ideal system the damping would be equally effective under each of these conditions. Differences in form may be attributed to differences in damping in this study. More accurate detail appears to be shown on the record of 400 pounds total mass. Paper speed is 25 mm. per second.

plethysmogram to the CDEF inscription of the displacement ballistocardiogram after footward mass movement of blood begins. There is, however, a time difference of 0.13 second between these recorded events (S_y and C).

A predictable effect on the recorded excursion of changing the total load of our heavy bed system by 100-pound increments with the same subject under resting conditions is shown in Fig. 7. The influence of changing the total load on the amplitude of the velocity and the bed displacement ballistocardiograms is significant. During the rapid displacement phase of cardiac ejection, the excursion "CD" reduces from about 0.0052 to 0.0037 inch and to 0.002 inch with addition of 100 and 200 pounds, respectively. This is an average change of 0.0015 inch of bed movement per addition of 100 pounds of mass including the

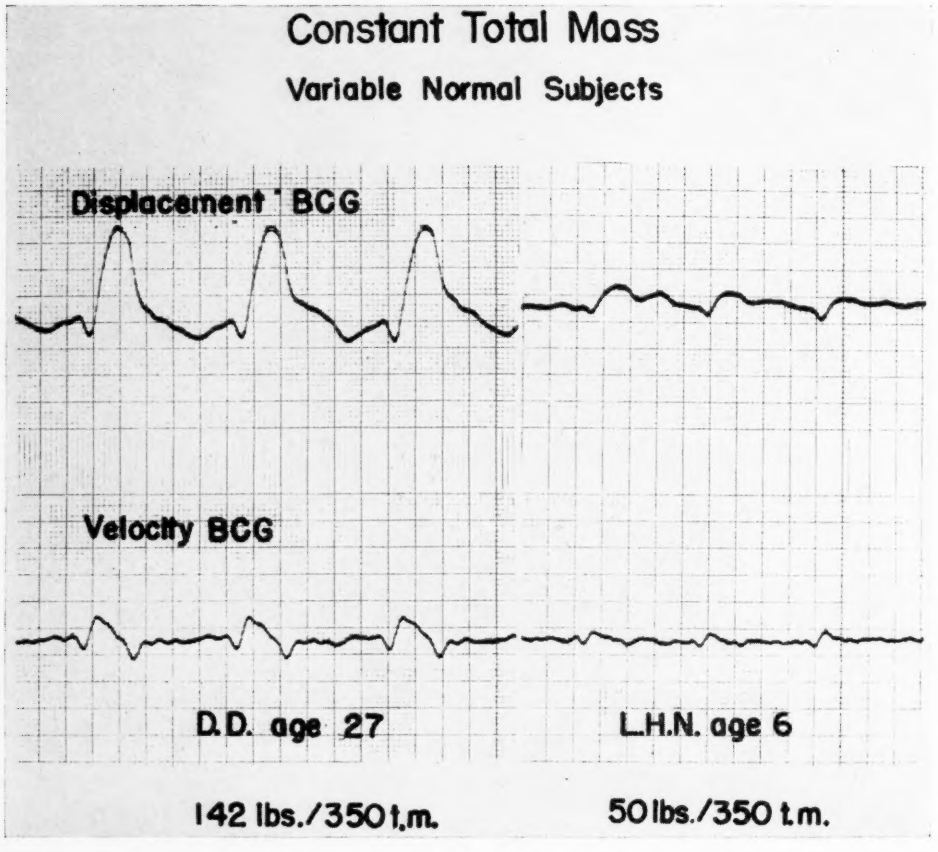


Fig. 8.—In this study the total mass of the bed plus each subject is constant and equal to 350 pounds. Great differences in amplitude of the displacement and velocity ballistocardiograms are noted between the normal subjects of 142 and 50 pounds, respectively. Paper speed is 25 mm. per second.

bed, subject, and added weights. During this deviation "CD," the calculated bed velocity at 200 pounds mass was about 0.026 inch per second, 0.185 inch per second for 300 pounds, and 0.0011 inch per second for 400 pounds of total mass carried. The average velocity decrement of 0.008 inch per second occurred for each 100 pounds added under these conditions. These effects showed as a decrement of about 4 to 5 mm. in the recorded position of the chief velocity peaks for each addition of weight shown in the velocity ballistocardiogram in Fig. 7.

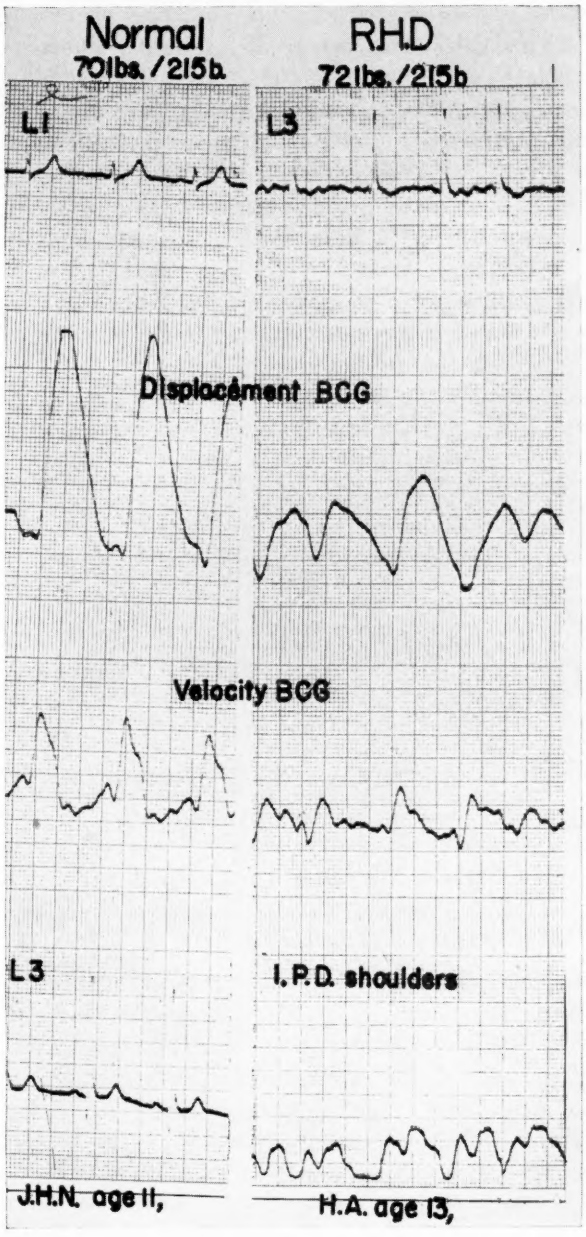


Fig. 9.—In this study we are dealing with normal and abnormal subjects of approximately the same weight on a constant mass pendulum ballistocardiogram of 215 pounds. Great differences are noted in the amplitude and configuration of these displacement and velocity ballistocardiograms, although the conditions of recording are equivalent in other respects. The significance of these differences is not yet fully understood but may serve as a valuable index of cardiovascular function. The paper speed is 25 mm. per second.

The effect of exchanging a normal subject of a given mass for another one having a different mass while retaining the same total mass of the system including the subject is shown in Fig. 8. The larger chief excursion "CD" is associated with the mature subject having greater weight. The smaller chief excursion "CD" is associated with the 6-year-old subject weighing 50 pounds. The difference in the amplitude of this footward excursion appears closely related to the relative difference in the inital force or energy expended as well as the mass displaced by the given beat against a fixed total mass. Similar inferences may be made from the relative study of the velocity of the bed during this phase and interval in the same record (Fig. 8).

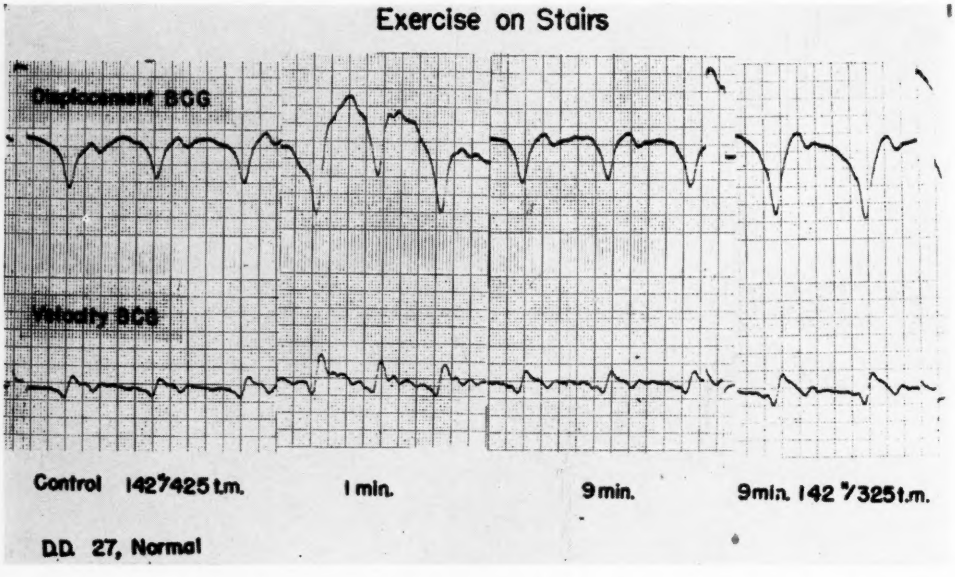


Fig. 10.—The effect of exercise in this case is to increase the amplitude and frequency of the displacement and velocity ballistocardiograms. The effect on the amplitude of the beats is somewhat comparable to reducing the total mass of the system by 100 pounds. This effect is shown on the right at 9 minutes after exercise. The paper speed is 25 mm. per second.

The relative depressing effect of rheumatic heart disease with decompensation upon the size of movements of the bed is compared in Fig. 9, with a normal subject of about the same weight with the same total mass to be moved in each case. The chief excursion "CD" varies with each beat in the case of auricular fibrillation. The longer the preceding pause, the larger is the chief excursion related to cardiac ejection in a footward direction. The normal 70 pound subject shows an average "CD" excursion about three times the amplitude of the diseased subject under otherwise similar conditions.

The effect of a physiologic change such as exercise on bed displacement may also be evaluated in terms of displacement or velocity of the bed. This is shown in Fig. 10. The frequency response characteristics of the bed, the pendulum period, the subcritical damping, and its total mass are essentially constant while the response varies with the thrust of each heartbeat or dynamic mass displacement. Exercise doubles the apparent displacement in each case

and also modifies the velocity. Rest restores the effect of displacement and velocity to the pre-exercise level. These primary changes may be compared to the effect of decreasing the load by 100 pounds, which is also shown.

DISCUSSION

The Newtonian law of dynamic equilibrium defining an equal and opposite measurable reaction to a given physical force is also the basis for all ballistocardiographs whether they are identified with force, velocity, or displacement phenomena. Burger and his associates[2] have expanded theoretically and experimentally the recoil effect of the body identified with each heartbeat as introduced by Gordon[25] and rediscovered by Henderson[1] from studies of the human body suspended on a low-frequency pendulum type of bed. Since the initial velocity of the entire damped system is zero and the center of gravity of the ideal system remains the same with different subjects, the first approximation of the rapid cardioballistic phenomenon may well be expressed by their derived equation

$$m_1x_1 + m_2x_2 = d \qquad (1)$$

in which x_1 and x_2 are the respective displacement of m_1 (total mass carried) and m_2 (blood mass) and d is a displacement constant. From further solution of differential equations of such a swing with a weak binding to the surroundings without springs and a low-frequency natural period, Burger and his associates[2] also concluded that the recorded dynamic displacement (x) of a large mass (M) is proportional to the displacements (Y) of the smaller mass of blood (M) within the system. Thus, equation

$$x = my/M \qquad (2)$$

defines the records of the dynamics reproducing the bed displacement from the center of gravity with each mass displacement of blood within the arteriovenous system.

The observed relation between the size of the recorded phasic displacement due to the heart and the numerical value of the total load of bed, subject, and weights is an inverse one. This was demonstrated in Fig. 7. The mathematical relation between the size of the recorded phasic displacement (x) of the bed and the numerical value of the diphasic mass displacement of blood (Y) from the resting center of gravity is a direct one. This probable direct relationship is apparent from the study of the amplitude of the cardiograms before and after exercise as shown in Fig. 10. At present one cannot define the value of this blood mass (m) or the distance (y) it traverses in a headward or footward direction. The record obtained is proportional to the right-hand portion of this equation and is the resultant of headward and footward mass movement (my). In this manner, the cardiogram record resembles the plethysmogram which is an arteriovenous volume difference. We do not propose to settle the empirical or quantitative relation between plethysmograms and displacement ballistocardiograms but we feel there is much to be gained by further correlation of these.

If the total load and damping in displacement ballistocardiograph remain constant in observations from subject to subject or in a given subject under altered hemodynamic conditions, the records of bed displacement from the center

of gravity become valuable indices of the relative amount of blood being moved headward and footward under these conditions. Some of the ballistic and plethysmographic phenomena we wish to evaluate independent of exercise are respiration, overweight, malnutrition, natural or induced bradycardia, tachycardia, stages of cardiac decompensation, and other functional and anatomical modifications of the heart and vascular system produced by surgical intervention or disease.[26]

Although our studies have been made with a heavy bed, we feel that the lightest bed[3] possible to withstand a load of 350 to 400 pounds should be most suitable to assure us of the best physical coupling between the patient and a constant mass system. This heavy load will introduce some lag in recording in comparison with the recording from a 4-pound wire suspension bed made by Rappaport. It will aid us, however, in stabilizing or designing a transducer system of ballistic movement and in correlating findings from one subject to another under similar conditions of damping and loading of the bed.

Our limited experience indicates that we have not sacrificed detail of configuration of the records just because the load is large (Fig. 7). The subject's own mass cannot be disregarded in any ballistic study of mass movements of blood and the counter movements by the body or attached devices. The diphasic ballistocardiogram looks different from the plethysmogram because it is responsive to differential headward and footward mass movement repeatedly within the same cardiac cycle, whereas the plethysmogram records the differential arteriovenous volume or cross section of a given segment and thus appears like a monophasic curve. If the headward mass movements of blood were rectified and immediately added in the same direction to the footward movements of such a modified displacement ballistocardiogram, it would more nearly simulate the form of the pulse volume curves of large body segments such as the torso or hand-to-feet plethysmograms. Such an instrument could be constructed and operated simultaneously with plethysmographs to study empirical and quantitative implications of segmental or mass movements of blood.

Although our studies have stressed the measurement and evaluation of changes on the basis of dynamic diphasic displacement of the bed from the center of gravity, the recorded changes in velocity of bed movement are also valuable. This has been expressed by an equation by Burger as

$$x = \frac{m}{\beta} \frac{dy}{dt} \qquad (3)$$

which indicates that the measure of displacement (x) is proportional to the velocity $\frac{dy}{dt}$ of the ejected blood (m). The symbol β (Greek letter beta) represents the value of the damping factor which corrects for the amplitude and phase distortion of the velocity ballistocardiograms.

In general it appears to the authors that the widely used acceleration type of ballistic method renders chiefly data on peak forces in the cardiac cycle. On the other hand, the low-frequency bed displacement methods give useful records from which the average acceleration ($\bar{a}$) and average displacement ($\bar{x}$) for the pulse cycle may be calculated. It follows that since we also know the total mass

(m) being moved we now have a measure of the force of the pulse and the work of the pulse against the given damping resistance for the pendulum bed. Thus,

TOTAL PULSE WORK $\simeq \bar{a}\ m\ \bar{x}$ of the table, subject and added weights. (4)

Factors such as forced inspiration favor a reserve of venous blood for ejection, whereas in forced expiration there is a minimum venous return and less blood for ejection. These observations explain reasonably well the changes in mean amplitude and form of the low-frequency displacement ballistocardiogram, during respiration. In effect we are always dealing with arteriovenous dynamics rather than primary cardiac or arterial dynamics in displacement ballistocardiography.

CONCLUSIONS

1. Satisfactory records of displacement and velocity ballistocardiograms were obtained when the total load of a low-frequency pendulum bed approached 450 pounds.

2. Comparison records of electrical impedance plethysmograms from the torso were made with simultaneous displacement ballistocardiograms of the bed.

3. A constant load displacement ballistocardiograph appears to have advantages of interpretation and technique in dynamic cardiovascular studies of various healthy and diseased subjects.

4. The mathematical formulations of Burger and his associates[2] in regard to cardioballistics appear to be applicable to the immediate problems of displacement ballistocardiography.

We are appreciative of the time and assistance which our voluntary subjects, Lee, Peter, and John Nyboer, patient subjects, and laboratory assistants, Edwin C. Thornton, Miss Dorothy Donnelly, and Dr. Robert E. DeForest, have given us in this project. Advice and construction of the plethysmograph were obtained graciously from Mr. Matthew Conrad of Philadelphia and Mr. Samuel Bagno of New York.

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A METHOD FOR OBTAINING IN VIVO SPECTRA OF BLOOD PIGMENTS

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OPTICAL methods were first applied in the study of blood pigments by Hoppe-Seyler in 1862.[1] He observed the two-banded spectrum of oxyhemoglobin. Stokes obtained the spectrum of reduced hemoglobin in 1865. Cherbuliez[2] determined the quantitative absorption curve of carbon monoxide hemoglobin in 1890. The fundamental studies concerning absorption spectra of blood pigments are reviewed by Heilmeyer.[3] All of these early studies used either whole blood or dilute alkaline blood solutions.

Vierordt,[4a, b] in 1875, found the absorption spectra of oxyhemoglobin in studies of light transmitted through human skin. He noted that a shift to the reduced hemoglobin curve followed suppression of the blood circulating through the transilluminated tissue area. Nicolai,[5a, b] in 1932, developed a technique for the exact in vivo measurement of absorption of trichromatic light through a fold of skin. The three lines used, 579, 577, and 546 mμ, were filtered from the mercury emission spectrum. By coincidence, these correspond to the absorption maxima of oxyhemoglobin.

In 1935, Kramer[6a-c] and Matthes and Gross,[7a-e] each working separately, originated a new technique which permitted estimation of oxygen saturation in flowing blood. These results were made possible by the simultaneous measurement of the absorption of two spectral regions in the same tissue area. These two spectral regions were obtained from a universal light source by the use of two filters, one red and the other infrared. The red filter transmitted all wave lengths above 600 mμ. The infrared filter transmitted from 750 to 900 mμ. Absorption in this infrared region is approximately the same for oxyhemoglobin and reduced hemoglobin (see Horecker[8]). Absorption in this region changes only with changes in the total amount of the two hemoglobin pigments and is independent of the degree of oxygenation. Horecker has determined an isobestic point of 800 mμ for the oxyhemoglobin and reduced hemoglobin system. In the red band, the absorption by the two pigments is quite different. Increases in reduced hemoglobin will reduce the absorption in this region. Thus the use of two filter-covered photocells permits determination of the ratio of oxyhemoglobin to reduced hemoglobin in a transilluminated tissue area through which blood is flowing.

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Filter oximeters operating on this principle have been developed by Millikan,[9a-c] Squire,[10] Goldie,[11] Wood,[12a, b] and others. Clinical oximeters are of two types. One utilizes a polystyrene blood cuvette joined to an intravascular cannula. Such an oximeter measures whole blood absorption. The second type transilluminates some tissue such as the pinna of the ear. This latter method, while far more convenient for many clinical applications, requires correction for absorption by tissues other than blood. Two methods of correction have been employed. The Millikan oximeter requires calibration in terms of known values of arterial oxygen saturation. Most of the other instruments are calibrated to read "zero" after the blood has been forced out of the area to be transilluminated. The simplest method for obtaining a bloodless ear appears to be the rubber diaphragm such as that described by Wood.[12a]

Wide-band filter oximeters, such as those currently used clinically, have the advantage of compactness. Small size is made possible by the use of a small light source and a barrier-layer photosensitive device. However, the use of wide-band filters introduces error and variability which is undesirable in applications requiring quantitative data. This is especially true in the fields of altitude physiology or cardiovascular diagnosis. The wide infrared band used in present-day oximeters stretches far on each side of the 800 mμ isobestic point. Thus, variations in a single pigment may produce slight differences in the absorption values. Quantitative application of the Beer-Lambert law to the red reading is not possible since the postulates on which the law is based do not hold over a wide spectral region. That the deviation from Beer's law can be ignored, if no greater accuracy than the present oximeters possess is desired, is validated by Paul.[13]

The authors are of the opinion that statistical analysis of absorption curves in the region from 400 to 1,000 mμ will indicate those wave lengths most suitable for the quantitative measurement of absorption differences between any two blood pigments. This would also permit some prediction of the error introduced by widening the band widths around such wave lengths of maximum differentiation.

Using a modified Wood technique, the authors have investigated a method for the in vivo examination of the total spectrum of blood pigments in the region from 400 to 1,000 mμ. The present method was developed to show the potential simplicity of the monochromatic source approach in vivo. It was not our intention at this time to attempt the quantitative determination of any particular pigment system. Rather, it was felt that this initial series of experiments should only demonstrate the potentiality of a monochromatic source system for the detection of in vivo changes in blood pigment concentration. In contrast to the oximeter work, we have chosen oxyhemoglobin and carbon monoxide hemoglobin, for our consideration. We feel that the data herein presented substantiate the validity of our approach, that the initial demonstration has been made, and that the problem now is one of instrumentation, not method.

METHODS

An instrument possessing a carefully calibrated monochromatic light source and balanced photoelectric measuring circuit was necessary. The Beckman Model DU spectrophotometer was chosen. Using rabbits as the experimental animal, only slight modification of the standard instrument became necessary. The two light apertures into the specimen chamber were covered with glass slides. The rabbits were held in special rabbit holders* which clamp the animal at the upper thoracic and lower lumbar levels. This held body movements to a minimum. A laparotomy towel with a small circular hole was thumbtacked over the head of the animal in such a way that the ears protruded through the opening while the face was held immobilized in a soft cork ring attached to the floor of the holder. The holder with rabbit in position was inverted and suspended by clamps from two ringstands. The entire unit was placed over the Beckman in such a way that one ear projected into the sample chamber of the instrument.

A device for pressurization of the ear was constructed from two finger cots, one inside the other, firmly attached by rubber bands to a length of pressure hose. The hose connected through a T-joint to a standard mercury blood pressure manometer and rubber bulb. All connections were sealed with vacuum grease. The pressure device was inserted parallel to the rabbit ear into the sample chamber. Thus the light passed from the monochromator through a glass slide, through the pressure cot, through the pinna of the ear, through the second glass slide, and finally into the phototube housing. Lateral portions of the chamber were filled with inflated finger cots in order to prevent the ear from moving. Strips of felt material and a rubber sheet were placed snugly around the rabbit ear and over the chamber. This prevented extraneous light from entering the sample chamber. Animals used in this experiment were albino rabbits† weighing from 3.5 to 4.9 kilograms.

After preliminary experimentation, it was decided to "zero" readings at 200 mm. Hg. It required from 15 to 30 seconds to obtain a constant zero point. The pressure within the cots was then reduced to 50 mm. Hg. This latter pressure was low enough to permit circulation and high enough to prevent change of position.

Carbon monoxide was administered in two ways. In the first series of experiments, carbon monoxide was mixed with air in a 37.3 liter chamber. This mixture was then circulated by means of a push-pull vacuum pump through a face mask worn by the rabbit. In the second series of experiments, pure carbon monoxide was administered intermittently throught a special face mask which was worn for only 15 seconds at a time. Carbon monoxide was reapplied at 3-minute intervals until the animal presented clinical evidence of the desired level of intoxication. Spectrophotometric readings were then taken.

The optical density of the finger cot at the expansions corresponding to various levels of pressure was studied. These findings are depicted in Fig. 1. The Beckman was zeroed on the finger cot expanded to a pressure of 300 mm. Hg. Two additional glass slides were placed in the chamber to simulate the thickness of the ear. Optical density readings were then taken from 380 to 1,350 mμ. A complete spectrum was recorded at each of five pressure levels; 250, 200, 150, 100, and 40 mm. Hg.

In the first series of animal studies, complete spectra of the ear were taken from 400 to 1,000 mμ. Eleven critical wave lengths were then chosen and only these wave lengths were used for the second series.

Fluctuations in the blood content of the transilluminated tissue made it necessary that all absorption data be interpreted relative to the absorption of one particular reference wave length, 490 mμ. This choice was made in view of the isobestic of the oxyhemoglobin and carbon monoxide hemoglobin system at 496 mμ, described by Horecker. It became technically more convenient to use 640 as the immediate reference point for the portion of the spectrum between 640 and 1,000 mμ. In the last series of experiments, readings at 490 or 640 were taken immediately before and after each reading at any other

*Developed at Kettering Laboratory, University of Cincinnati.
†Purchased from New Delhi Rabbitry, Cincinnati, Ohio.

wave length. This allowed for the slight variations in circulating blood volume within the pinna region which might occur over short time periods and resulted in a slight increase in the accuracy of the method.

An attempt was made in the second series to correlate absorption changes with blood carbon monoxide levels. Carbon monoxide hemoglobin concentration was determined by the use of the Huffner and Heilmeyer points, 576/560 and 541/560, respectively, on blood samples diluted 1:100 with 0.4 per cent ammonia solution.

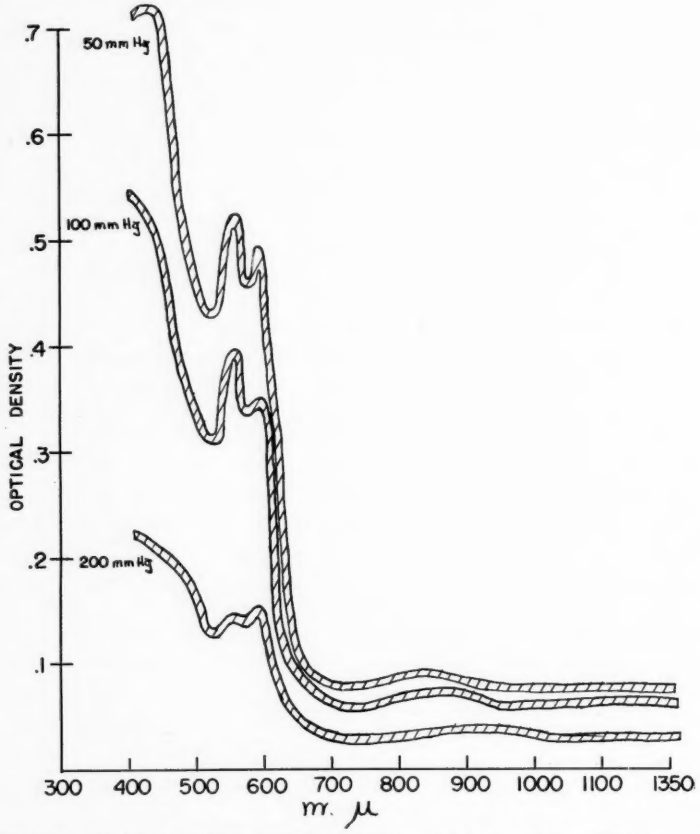

Fig. 1.—Variation of blood spectra with pressure. Ear in place; differences in absorption spectra caused by differences in pressure. (Instrument zeroed at 300 mm. Hg pressure.)

DISCUSSION

Fig. 1 depicts the absorption spectra obtained by exerting each of three different pressures upon the ear. The measurements were made serially after zeroing the instrument while the finger cot registered 300 mm. Hg pressure. The ear contains a small amount of blood at 200 mm. Hg. It was felt that this was sufficiently small an amount to permit the use of 200 mm. as the bloodless ear base line. Higher pressures were more difficult to stabilize, more discomforting to the animal, and put more stress on the pressure device air seals. No attempts were made during this study to correlate intracot pressures with actual pressures against the ear. The absorption curves at 50 and 100 mm. Hg both appeared to resemble the absorption curve for whole

oxygenated blood given by Heilmeyer.[3] This characteristic pattern was found in all of our normal blood determinations, as evidenced by curves III and IV. The very small differences between our curves and those of Heilmeyer would appear to be in keeping with the expected finger cot absorption.

The data of Fig. 1 would suggest that either 50 or 100 mm. Hg might have been used as a pressure level for the blooded ear. It was felt that the 100 mm. pressure is less desirable in view of its more marked restriction of blood flow. This is evidenced by the difference between the 50 mm. and 100 mm. curve abscissas. It is also suggested by the low 580 peak in the 100 mm. curve. This might indicate a shift from oxyhemoglobin to reduced hemoglobin because of hindered circulation. The 50 mm. pressure was therefore selected.

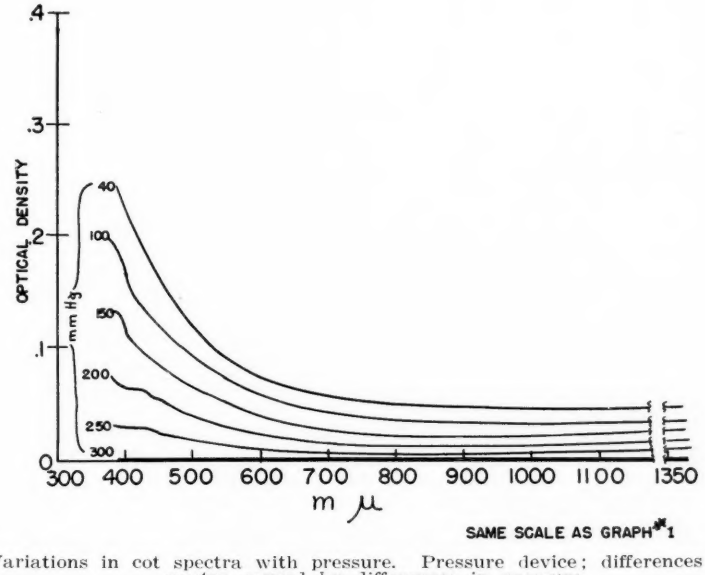

Fig. 2.—Variations in cot spectra with pressure. Pressure device; differences in absorption spectra caused by differences in pressure.

Fig. 2 shows the absorption spectra of the finger cot device alone at five pressures below 300 mm. Hg. Since this absorption remained the same for both carbon monoxide and normal respiration studies, no correction was applied in routine calculations. Figs. 1 and 2 have been drawn to the same scale. A comparison of the two figures indicates that an appreciable portion of the total absorption above 700 mμ is due to the finger cot. If the finger cot absorption correction were included in the histograms of Fig. 7, the difference between normal and carbon monoxide containing blood would become even more evident.

Figs. 3 and 4 picture the in vivo absorption spectra of blood during normal respiration for experimental Series 1 and 2, respectively. The average of 5 values in Series 1 and of 6 values in Series 2 is drawn as the mid-line of an area shaded to represent the average deviation from this line. Only one study was done for values below 450 mμ or above 950 mμ. The

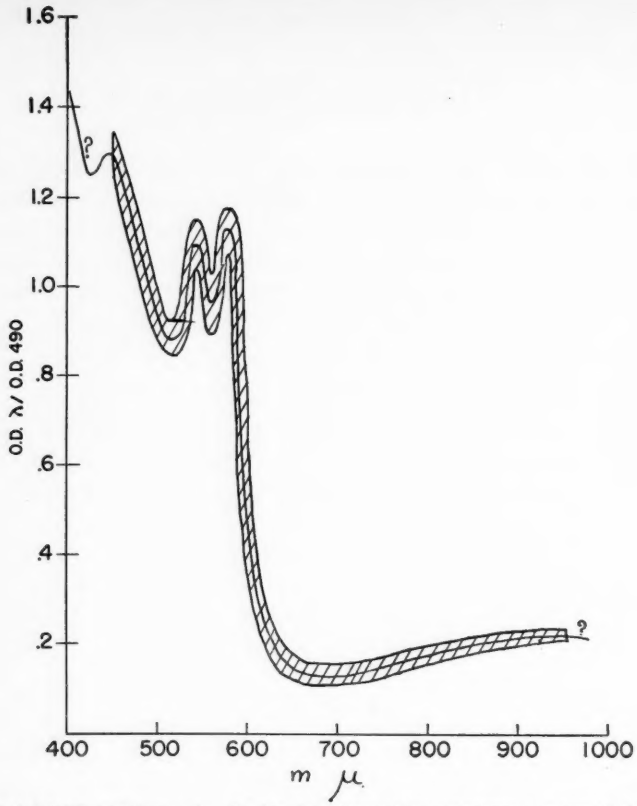

Fig. 3.—Normal blood spectrum I. Series 1; absorption spectrum, normal respiration. (Midline is average; shaded portion is standard deviation.)

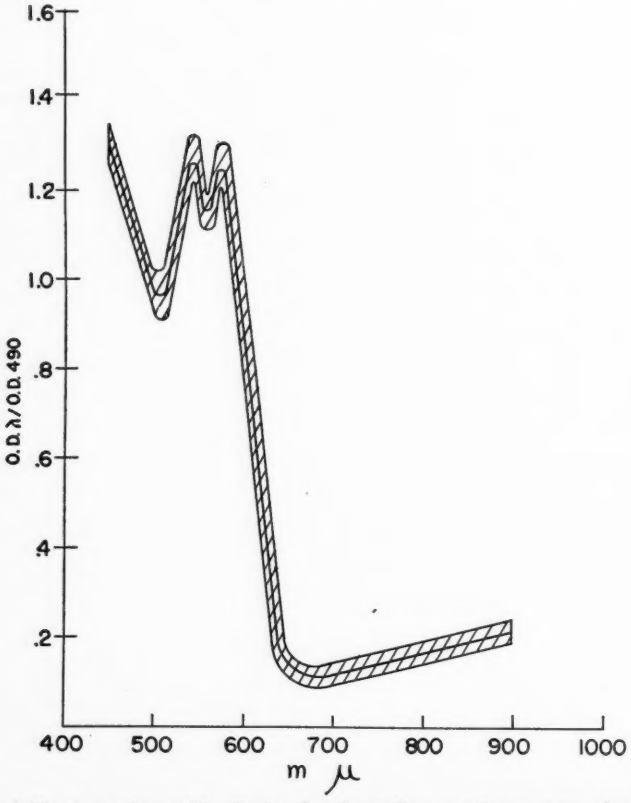

Fig. 4.—Normal blood spectrum II. Series 2; absorption spectrum, normal respiration. (Midline is average; shaded portion is standard deviation.)

question mark in these regions of the illustration indicate that they should not be interpreted with the same statistical implication as the remainder of the spectrum.

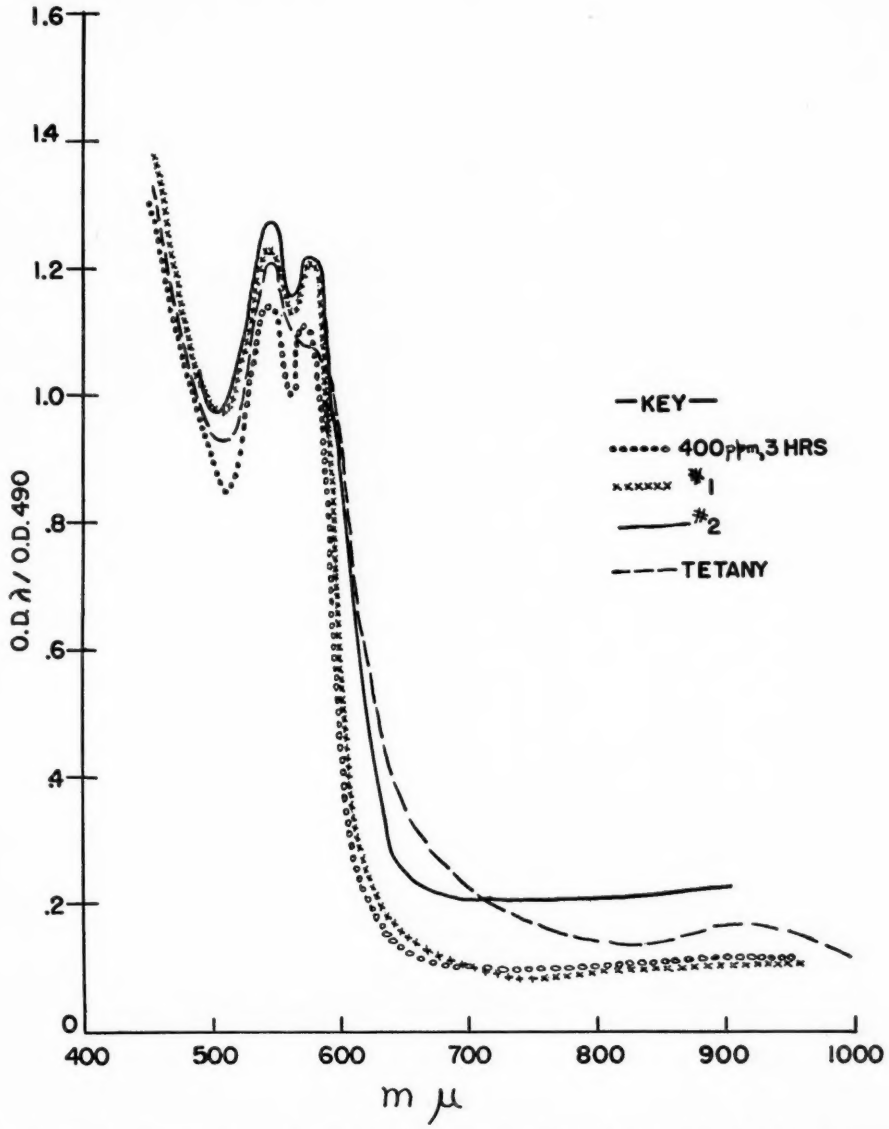

Fig. 5.—Carbon monoxide blood spectra I. Series 1; absorption spectra during exposure to carbon monoxide.

The resemblance of these curves to that of Heilmeyer[3] for oxygenated blood is apparent. Unfortunately, Heilmeyer does not give values for the spectral region above 650 mμ. Horecker[8] has determined the absorption by solutions of pure blood pigments for the region from 420 to 1,000 mμ. The portion of our curves above 600 mμ closely resembles the corresponding

region of Horecker's oxyhemoglobin curve. The curves for hemoglobin and oxyhemoglobin also closely resemble those of Drabkin and Schmidt.[14] These workers placed a cuvette into the femoral arteries of dogs. The cuvette was then transilluminated using a Bausch and Lomb spectrophotometer. The

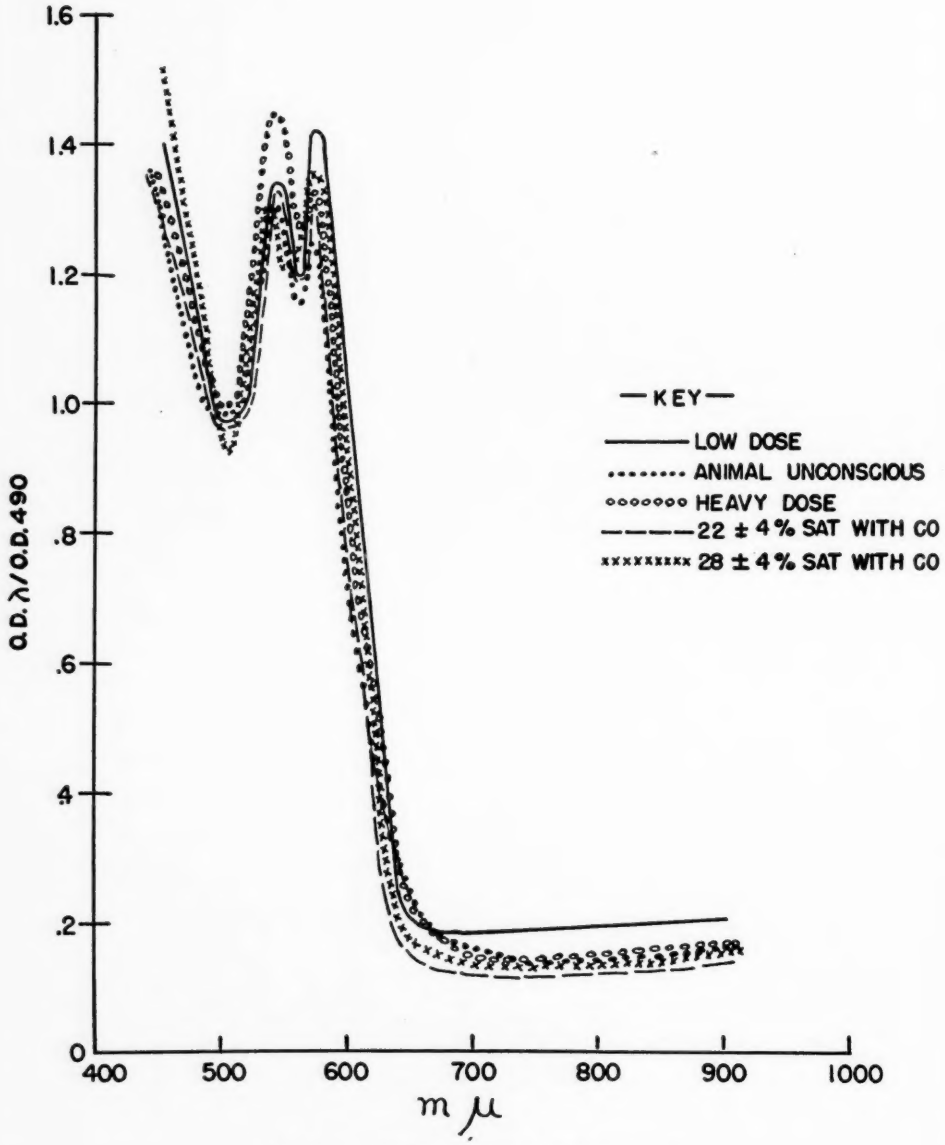

Fig. 6.—Carbon monoxide blood spectra II.

cuvette received blood from the femoral artery and returned the blood to the same vessel. Their studies were made in the range from 505 to 630 mμ. Measurements were taken at 505, 542, 562, 578, 600, and 630 mμ. It would

be anticipated that the present results of actual in vivo study would resemble those obtained by their method which utilized a cuvette containing actively circulating blood, and there is no indication to the contrary.

The markedly positive slope of the absorption curve in the region beyond 700 mm. is readily apparent. This is in marked contrast to the flattened

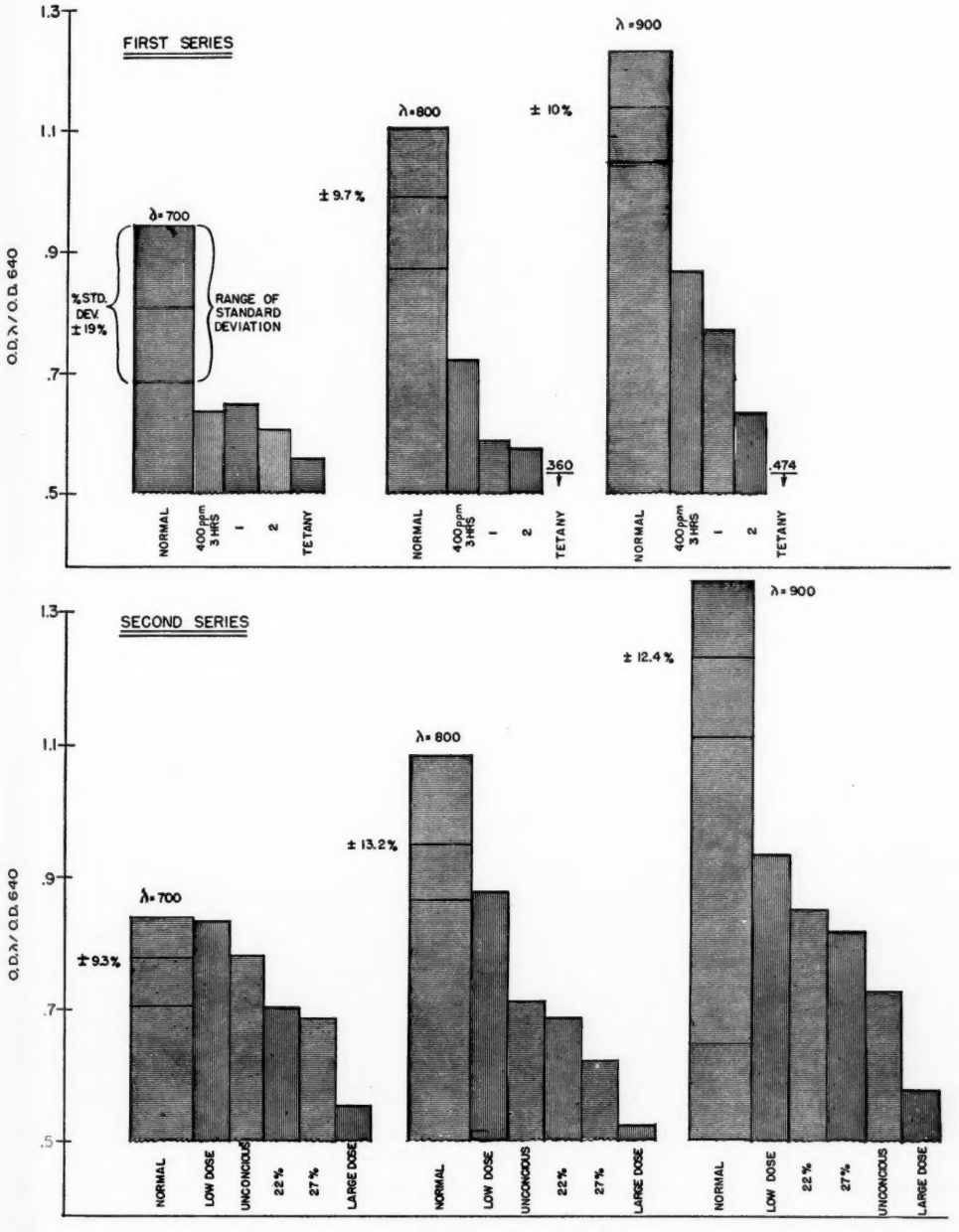

Fig. 7.—Histograms illustrating the significance of changes in spectra brought about by coexposure.

appearance of the corresponding area in the carbon monoxide exposed animals, as shown in Figs. 5 and 6. These two figures represent the in vivo absorption spectra of blood after exposure of the animals to carbon monoxide.

The clinical symptoms, parts per million carbon monoxide inhaled, or blood carboxyhemoglobin concentration are listed for each curve on Figs. 5 and 6 below the corresponding bar on the histograms of Fig. 7. The first series, thus, consisted of animals:

1. Exposed to 400 ppm for an hour prior to reading, also exposure was continued during readings.

2. Exposed to 50 c.c. pure CO (No. 1).

3. Exposed twice to 50 c.c. pure CO (No. 2).

4. Exposed to a few parts per thousand CO until a condition of tetany was evident.

The second series consisted of animals treated as follows:

1. Exposed to three 15-second periods during which pure CO was breathed. The animal was allowed to respire normally for a minute between each exposure. This is called "light dose" on the graphs.

2. and 3. More periods of exposure were used in each case than in *1*, and blood samples were taken for CO hemoglobin analysis. The results were 22 per cent and 27 per cent saturation, respectively.

4. Animal exposed to CO in 15-second doses until unconscious.

5. Pure CO administered at intervals until animal was barely alive, ears flushed, shallow breathing, etc. Animal denoted as "heavy dose" on the graph.

No attempt was made in the first series of experiments to correlate specific dosage levels and clinical manifestations with specific degrees of alteration in spectra.

The Series 2 absorption curves were more closely related to the reference absorption point, as indicated previously. An attempt was made to correlate dosage, clinical syndrome, alterations in spectra, and, in two instances, blood carbon monoxide hemoglobin.

Comparison of Figs. 5 and 6 with Figs. 3 and 4 brings out characteristic differences produced by the exposure to carbon monoxide. These include:

1. A shift in the minimum from 510 to 500 mμ. Since the reference point, 490, is on a negative slope of the curve, this also tends to raise the entire carbon monoxide curve.

2. Flattening of the 700 to 900 region.

The second finding suggests a means for measuring the concentration of carbon monoxide hemoglobin through analysis of changes in the 700/640, 800/640, and 900/640 absorption ratios. The histograms of Fig. 7 reveal the results of this approach. There is a definite correlation between exposure to carbon monoxide and diminution of the 900/640 optical density ratio. Quantitative correlations between specific blood levels and specific deviations in curves have not been attempted. It would be suspected from these crude studies that such correlations exist. Changes in the 900/640 ratio appear

to be much more marked than those in the 800/640 ratio (Tables I and II). It would thus appear that the modification of existing oximeters to utilize the 900/640 ratio might considerably augment the potentiality of such instruments for the measurement of carboxyhemoglobin saturation. The unpublished studies of Harper[15] were done with the filter oximeters of the University of Chicago Clinics. Such instruments are based upon the standard filter systems. Rabbit blood pigments do not appreciably vary from human blood in their absorption spectra. The relatively minor species differences are reviewed in Prosser and associates.[16]

TABLE I. STANDARD DEVIATIONS FOR HISTOGRAMS. FIRST SERIES

FIRST SERIES	700	800	900
Mean	0.802	0.983	1.220
Standard deviation	0.152	0.095	0.122
Per cent deviation	19	9.7	10
Twice standard deviation	0.304	0.190	0.244
Mean minus twice standard deviation	0.498	0.793	0.976
Highest CO value	0.647	0.714	0.857
Lowest CO value	0.553	0.368	0.474

TABLE II. STANDARD DEVIATIONS FOR HISTOGRAMS. SECOND SERIES

SECOND SERIES	700	800	900
Mean	0.776	0.980	1.232
Standard deviation	0.072	0.129	0.154
Per cent deviation	9.3	13.2	12.4
Twice standard deviation	0.144	0.258	0.308
Mean minus twice standard deviation	0.632	0.722	0.924
Highest CO value	0.840	0.880	0.940
Lowest CO value	0.560	0.529	0.587

Figs. 3 to 6 demonstrate the feasibility of utilizing monochromatic light, or restricted band widths, for the in vivo investigation of blood pigments. The application of the equipment to human subjects necessitates minor modification of the spectrophotometer. The phototube must be separated from the monochromator by about a foot. Each may be fastened to a common base which is so shaped as to leave room for the head. A simple closed optical path may be constructed of tubes reaching from both light apertures almost to the center of the space separating the Beckman components. A glass plate at the end of each tube would provide a solid surface against which the ear would be compressed. A diaphragm similar to that of the Wood oximeter may be placed over the forward tube. The increased length of the light path would, of course, result in reduction of light intensity. Considerations of such instruments are still quite preliminary. However, it is probable that such an instrument would have to be zeroed with the sensitivity control. The absorption properties of the human ear may make quantitative readings below 600 mm. extremely difficult. However, the region from 600 to 1,000 mm. offers considerable information. Our studies in the region above 1,000 mm. are still preliminary, but they suggest that this region also may be of value.

The clinical utilization of a modified spectroscope for dilution curves, analogous to the dye dilution methods of Nicholson, Wood, and Burchell[12c] would necessitate automatic recording systems.

It would appear to us that total spectral methods will probably remain as a research tool for the determination of wave lengths of maximum utility for any particular system. Clinical instruments could then be developed as simple double-beam monocromators, coupled with barrier layer photocells and standard recording systems, which might then be utilized for trans-illumination or lateral transmission utilization.

SUMMARY

1. A method is described for in vivo measurement of the continuous spectrum of rabbit blood. This method is based upon modification of a Beckman spectrophotometer to permit its utilization in a manner similar to the Wood ear oximeter.

2. Spectra were obtained under normal respiratory conditions in a series of 11 rabbits. Spectra were obtained after carbon monoxide inhalation in a series of rabbits. Significant deviations from normal in the carbon monoxide curve were observed.

3. Statistically significant changes in the 900/640 ratio of optical densities brought about by carbon monoxide exposure were found. These changes correlated with clinical syndrome.

4. Modifications of existing instruments for possible clinical application in human subjects were briefly considered.

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INVESTIGATION OF THE METHODOLOGIC ERROR IN BRONCHOSPIROMETRY

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WITH the advances of modern thoracic surgery, the operative risk has been reduced to a minimum and increasingly serious conditions have become accessible to surgical treatment. This progress is due in no small measure to increased knowledge of the pathophysiology of pulmonary diseases and to enhanced facilities for preoperative evaluation of any ventilatory insufficiency that may occur. Today, bronchospirometry is an important routine feature of this functional analysis. Moreover, it is the subject of a quite extensive literature, which was recently reviewed by Gaensler.[1] Nevertheless, no analysis of the methodologic error in bronchospirometry appears to have been reported hitherto.

MATERIAL

The present investigations were included in the routine functional tests prior to evaluation of the operative indications in cases of pulmonary tuberculosis. A total of 75 examinations were carried out in 74 patients (one being examined postoperatively also), and no selection occurred. The series includes, as will be seen from Table I, both cases with a normal distribution of the relative function and cases with pronounced ventilatory insufficiency of one side or the other. The examinations were not associated with any complications aside from slight transient bronchitis symptoms in some cases.

METHOD

The tests were conducted with a "Lundia" double spirometer of modified Knipping type, in conjunction with Carlen's twin-channel catheter. The patients were examined in the mornings, before they had eaten, and the premedication consisted of 0.10 Gm. pentobarbital sodium two hours before the test as well as 0.01 Gm. morphine and 0.001 Gm. atropine subcutaneously three-quarters of an hour before. Mucosal anesthesia was accomplished with a solution containing 20 parts 1 per cent tetracaine and 1 part Adrenalin 1:1,000. The pharynx was first sprayed with this solution; then about 3 ml. was administered via indirect laryngoscopy for anesthesia of the trachea and bronchi.

The largest Carlen's catheter that was judged to be insertable without difficulty was then advanced, under indirect laryngoscopic control, until the carina was felt as a springy resistance against the hook. After inflation of the balloon-cuffs, the catheter was connected to the spirometer, the gasometers of which were filled with oxygen and the system checked for any leaks. The oxygen intake and tidal ventilation were then recorded for five minutes, after which three determinations were made of the vital capacity. Of the vital capacity determinations, those showing the highest values were used.

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TABLE I. PER CENT OF TOTAL OXYGEN INTAKE, MINUTE VENTILATION, AND VITAL CAPACITY FOR LEFT LUNG AT FIRST (I) AND SECOND (II) DETERMINATIONS

PORTION OF BILATERAL FUNCTION CONTRIBUTED BY LEFT LUNG (%)	NUMBER OF CASES					
	OXYGEN CONSUMPTION		MINUTE VENTILATION		VITAL CAPACITY	
	I	II	I	II	I	II
0-10	–	–	2	1	–	–
11-20	4	2	2	2	2	3
21-30	11	13	9	12	9	9
31-40	15	13	16	12	15	14
41-50	21	21	21	21	19	20
51-60	11	12	13	15	17	18
61-70	5	5	7	7	4	3
71-80	5	7	5	4	5	4
81-90	3	2	–	1	1	1
91-100	–	–	–	–	–	–
Total number of cases	75	75	75	75	72	72
Mean per cent of bilateral value for left lung	45.827	46.880	45.227	45.680	45.722	46.375

Following this initial investigation, the balloons were deflated and the catheter drawn up 10 to 12 cm.; it was then relowered into positions, the balloon-cuffs reinflated and the previously stated determinations repeated.

TABLE II. DISTRIBUTION OF CASES AS TO DIFFERENCE BETWEEN FIRST AND SECOND DETERMINATIONS FOR LEFT LUNG IN PER CENT OF TOTAL FUNCTION

DIFFERENCE IN PER CENT OF TOTAL BETWEEN FIRST AND SECOND DETERMINATION	NUMBER OF CASES		
	OXYGEN CONSUMPTION	MINUTE VENTILATION	VITAL CAPACITY
–18		1	
15		1	
12	1		
11	1		
10	1		1
7	3	2	2
6	2	1	
5	1	3	
4	4	7	2
3	2	6	2
2	7	7	3
– 1	13	8	23
0	12	9	19
+ 1	14	4	13
2	5	10	4
3	2	3	
4	1	1	1
5		2	
6	2	4	1
7			1
8	1	1	
9		1	
10	1	2	
11		1	
13		1	
14	1		
+29	1		
Total number of cases	75	75	72

RESULTS

Since the tests were generally completed within the course of half an hour, variations in the patient's condition could scarcely have influenced the

results; moreover, statistical analysis showed no systematic difference between the two series of tests (the mean difference did not deviate significantly from zero) in respect of any of the three factors investigated.

In the statistical analysis, the methodologic error was computed from the difference between the first and second investigation, the respective percentages, for each lung, of the total function being taken as unity.

Table II shows the distribution of the differences between the two investigations with respect to oxygen consumption, minute ventilation, and vital capacity for the left lung.

From Table III, showing the statistical data that were computed, it will be seen that with the method described previously, the mean error in determination of the oxygen consumption for each lung separately amounts to 3.8 ± 0.31 per cent of the total oxygen consumption. As regards the minute ventilation, the mean error for the respective lungs is 3.5 ± 0.29 per cent of the total minute ventilation; but, in the case of the vital capacity, it amounts to only 1.7 ± 0.14 per cent of the total vital capacity.

TABLE III. STATISTICAL EVALUATION OF METHODOLOGIC ERROR

	NUMBER OF DOUBLE DETERMINATIONS (n)	MEAN DIFFERENCE BETWEEN DOUBLE DETERMINATIONS ± S.E. OF MEAN DIFFERENCE ($\bar{d} \pm \Sigma_{\bar{d}}$)	S.D. OF SINGLE DETERMINATION ± S.E. OF S.D. ($S \pm \Sigma_S$)
Oxygen consumption	75	0.15 ± 0.62	3.8 ± 0.31
Minute ventilation	75	0.43 ± 0.58	3.5 ± 0.29
Vital capacity	72	0.26 ± 0.28	1.7 ± 0.14

SUMMARY

In 74 cases of pulmonary tuberculosis, 2 series of 75 bronchospirometric determinations of oxygen consumption, minute ventilation, and vital capacity were carried out and the results compared. The methodologic error in bronchospirometric tests was then calculated in respect to these three factors. Expressed in percentage of the total function, it was found to be 3.8 ± 0.31 per cent for oxygen consumption, 3.5 ± 0.29 per cent for minute ventilation, and 1.7 ± 0.14 per cent for vital capacity.

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POTENTIATION OF THE CURATIVE ACTION OF PRIMAQUINE IN VIVAX MALARIA BY QUININE AND CHLOROQUINE

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PRIMAQUINE, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline, was introduced as a curative antimalarial drug for vivax infections in 1950.[1] In carefully controlled clinical experiments conducted on inmate volunteers at Stateville Penitentiary by University of Chicago and Army medical investigators, it was found to be several times as active and to have a more slowly rising toxicity curve in Caucasian subjects than the most promising analogues of pamaquine previously tested. From preliminary studies it seemed probable that many naturally acquired infections could be cured by considerably smaller doses than those required to cause eradication of the disease in the original experimental subjects who were heavily infected with the Chesson strain of Southwest Pacific malaria. These observations have been confirmed as a result of laboratory and field studies by many investigators.[2–4] Primaquine will safely cure Korean vivax malaria, as manifested in returning veterans, in single daily doses which are well tolerated by both Caucasians and Negroes.[5–11] Primaquine is active against the early fixed-tissue stages (primary exo-erythrocytic forms) of *Plasmodium vivax*[12] as well as the late or persisting fixed-tissue stages (secondary exo-erythrocytic forms) of this parasite.[1] Although it has greater activity against the asexual blood forms (trophozoites) than does any of the other 8-aminoquinolines tested, the degree of activity is too low to establish complete eradication of trophozoites in all infected subjects by dosages that are nontoxic.[2, 13] For this reason primaquine has been used concurrently with either of three suppressive drugs, quinine, chloroquine (Aralen), or amodiaquin (Camoquin) in curative therapeutic regimens.† In addition to its direct effect on the asexual blood stages, or trophozoites, the concurrent suppressive drug could have a potentiating effect on the curative action of primaquine. The reason for entertaining this possibility rests on the similarity in chemical structure and

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†Concurrent quinacrine (Atabrine) and primaquine administration is contraindicated because quinacrine potentiates the toxicity of 8-aminoquinolines.

pharmacologic action of pamaquine and primaquine. A few experiments conducted in nonendemic areas under controlled hospital observation suggest potentiation of pamaquine by quinine. These experiments, however, cannot be considered definitive because the individual groups treated were either small or the different test groups lacked adequate controls.[14-16] The possibility of pontentiation of the action of any 8-aminoquinoline against tissue stages by concurrent administration of quinine, therefore, still remains a matter of uncertainty.

Aim and General Design of Present Experimental Study.—The investigations reported in this communication were set up under rigidly controlled experimental conditions in order to establish firmly whether either quinine or chloroquine potentiates the action of primaquine against the late tissue stages of vivax malaria. To establish this point, three groups of nonimmune white volunteers were infected by the same mosquitoes and were treated during primary attacks by three different schedules of drug administration: (1) quinine followed by primaquine,* (2) quinine concurrently with primaquine, and (3) chloroquine concurrently with primaquine.

Rationale of the Experiment.—If quinine does not potentiate the action of primaquine against the tissue stages of vivax infections, the relapse rates after treatment by the first two schedules should be of the same order of magnitude. If, on the other hand, quinine acts synergistically with primaquine against late tissue stages, a much higher percentage of individuals should be cured when the drugs are administered concurrently (Schedule 2) than when primaquine administration follows that of quinine (Schedule 1).

Unlike quinine, chloroquine persists in the blood for many days. Therefore, evaluation of potential synergism between chloroquine and primaquine, administered concurrently as in Schedule 3, can best be made by comparing the cure-rate subsequent to their combined administration with the cure-rates after the two schedules wherein quinine was employed. If chloroquine potentiates primaquine, significantly more cures should occur after therapy according to Schedule 3 than after therapy according to Schedule 1.

METHODS AND PROCEDURES

Fifty-seven Caucasian volunteers from the general inmate population at the Illinois State Penitentiary (Stateville Branch) were divided into three groups of 19 men, each group to be treated by one of the three experimental therapeutic schedules. None of the men had a history of exposure to malaria. The malaria used in these experiments was the Chesson strain of *Plasmodium vivax* and has been used repeatedly in the past because of its ability to relapse early. The experimental volunteers, one from each of three groups, were bitten by the same 10 *Anopheles quadrimaculatus* mosquitoes infected with malaria. The three men were infected by intermittent biting of the mosquitoes in a systematic manner. For instance, mosquito No. 1 first fed on a volunteer in Group 1, next on an individual in Group 2, and finally was allowed

*The reverse order of giving drugs was considered but was rejected because primaquine will not invariably cause complete eradication of trophozoites.[2, 13] In experiments involving pamaquine and quinine,[15] potentiation of curative action was not apparent when quinine was administered after pamaquine in a small number of patients treated. Administration of the 8-aminoquinolines prior to quinine would appear to have no advantage and might even induce an additional factor hard to evaluate, namely, the development of partial immunity to erythrocytic forms during therapy.

to bite to completion on a subject in Group 3. The second mosquito infecting these three men began with the volunteer in Group 2, then bit the man from Group 3, and finally completed biting on the man in Group 1. The third mosquito first infected the subject in Group 3, then bit the man in Group 1, and finally fed out on the man in Group 2. This system was repeated again by mosquito No. 4 which began with the subject in Group 1, and so on. The men were rebitten until each man had received 10 infective bites. This elaborate technique for infecting volunteers was employed in order to assure the greatest possible uniformity in the sporozoite doses that were received by individuals in the three groups.

As a measure of the intensity of the infection that each man received, the salivary glands of each mosquito were dissected after it had fed on three men, one from each group. The mosquito infections were graded 1 plus if their salivary glands contained between one and ten sporozoites, 2 plus if they contained 11 to 100 sporozoites, 3 plus if 101 to 1,000 sporozoites were present, and 4 plus if more than 1,000 sporozoites were found. The maximum mosquito-infectivity under the semiquantitative method used to estimate sporozoite dosage is 40 plus, while the lowest for ten infected mosquitoes is 10 plus.

It is certain that the infected volunteers presented a severe challenge to cure because they all exhibited parasitemia and fever before the fourteenth day after inoculation by the mosquitoes, and often earlier.[17, 18]

Parasite counts were performed daily after the eighth day by the technique of Earle and Perez.[19] In order to reduce the development of immunity to a minimum, treatment was begun immediately after patients developed a fever of 102° F. and parasites had been demonstrated on two successive days. Details of procedures during the follow-up period have been described elsewhere.[20]

SCHEDULES OF DRUG ADMINISTRATION

All patients were treated during their primary clinical attacks on one of the following drug regimens:

Schedule 1 (Quinine Followed by Primaquine).—Two grams of quinine sulfate were administered in six divided doses daily, for 14 days, followed after an interruption of 48 hours by 15 mg. of primaquine base, in six divided doses daily, for an additional 14 days. This large dose of quinine was used because trophozoite recrudescence has been observed following blood inoculation on dosages as large as 1.0 Gm. of quinine sulafate administered for 14 days.[2]

Schedule 2 (Quinine Administered Concurrently With Primaquine).—Two grams of quinine sulfate were administered, in six divided doses daily, for a total of 14 days. With each dose of quinine, primaquine was also administered for a total of 15 mg. base each day. The total duration of combined drug administration was two weeks.

Schedule 3 (Chloroquine Administered Concurrently With Primaquine).—Fifteen milligrams of primaquine base was administered, in six divided doses daily, for two weeks. During the first 24 hours of primaquine therapy, 1 Gm. of chloroquine base was administered as follows: an initial dose of 0.6 Gm. base followed in 24 hours by 0.4 Gm. This dose of chloroquine was chosen because 1 Gm. of chloroquine base administered during the first 24 hours has invariably terminated trophozoite infections due to the Chesson strain of *Plasmodium vivax* in the extensive experience that we have had with this suppressive regimen.[2]

RESULTS

Only 21 per cent of the patients who were treated during the primary attack according to Schedule 1 were cured (Table I). The cure-rate following Schedule 1 served as a control for Schedules 2 and 3, and represents the simple additive effect of two drugs, one active against blood forms, the other active against tissue forms. Synergism between quinine and primaquine against tissue

forms was impossible on the first schedule of drug administration because an interval of sufficient length to allow complete elimination of quinine from the body intervened before primaquine was administered.[21] The possibility that the parasitemias in the treatment failures on Schedule 1 represented recrudescences of partially suppressed erythrocytic forms (trophozoites) rather than true relapses originating from tissue stages is virtually eliminated by the late

TABLE I. POTENTIATION OF THE CURATIVE EFFECT OF PRIMAQUINE BY CONCURRENT ADMINISTRATION OF QUININE OR CHLOROQUINE

		DRUG SCHEDULE NO. 1		DRUG SCHEDULE NO. 2		DRUG SCHEDULE NO. 3	
		QUININE FOLLOWED BY PRIMAQUINE		QUININE CONCURRENTLY WITH PRIMAQUINE		CHLOROQUINE CONCURRENTLY WITH PRIMAQUINE	
	INFECTIVITY OF MOSQUITOES*	QUININE SULFATE, 2.0 GM. FOR 14 DAYS FOLLOWED AFTER 48 HOURS BY PRIMAQUINE, 15 MG. IN 6 DIVIDED DOSES FOR 14 DAYS		QUININE SULFATE, 2.0 GM. CONCURRENTLY WITH PRIMAQUINE, 15 MG. IN 6 DIVIDED DOSES FOR 14 DAYS		PRIMAQUINE, 15 MG. IN 6 DIVIDED DOSES FOR 14 DAYS WITH 1.0 GM. CHLOROQUINE DURING THE FIRST 24 HOURS OF PRIMAQUINE THERAPY	
GROUP NO.	PLUSES	DAYS TO RELAPSE†	FOLLOW-UP†	DAYS TO RELAPSE†	FOLLOW-UP†	DAYS TO RELAPSE†	FOLLOW-UP†
1	27	56	---	--	367	---	366
2	15	--	365	--	372	184	---
3	29	52	---	--	366	---	373
4	30	43	---	--	367	---	365
5	32	--	365	--	373	---	166
6	31	68	---	--	369	46	---
7	33	92	---	--	250	---	369
8	27	--	366	--	370	---	310
9	30	68	---	--	365	44	---
10	32	76	---	--	171	---	365
11	29	47	---	--	362	---	364
12	32	49	---	--	231	---	361
13	34	53	---	--	182	47	---
14	28	65	---	--	365	---	365
15	25	50	---	--	196	61	---
16	31	--	114	--	344	---	288
17	28	45	---	17	---	---	342
18	18	67	---	--	223	---	325
19	25	32	---	--	322	---	321
	Per cent cured	21% (4/19)		95% (18/19)		74% (14/19)	

*Highest possible infectivity is 40, lowest possible is 10.
†Days to relapse and follow-up were figured from last day of primaquine therapy.
Note: The three cases in each horizontal row were bitten by the same 10 infected mosquitoes on the same day. Probability that cure rates are due to chance alone are:
comparing drug Schedules 1 and 2, <0.001
comparing drug Schedules 1 and 3, <0.01
comparing drug Schedules 2 and 3, 0.1–0.2.

occurrences of all parasitemias. In a study of the suppressive action of primaquine in blood-induced infections, trophozoite recrudescences have invariably occurred before 30 days after discontinuing the drug.[2] In extensive observations on blood-induced malaria, Chesson strain, during World War II, Shannon and his co-workers considered that, from a practical standpoint, a 21-day follow-up period after therapy with drugs that were quickly eliminated from the body was sufficient to evaluate trophozoiticidal action.[22]

Much higher cure-rates were obtained on Schedule 2 (95 per cent) and Schedule 3 (74 per cent), wherein quinine and chloroquine, respectively, were

administered concurrently with primaquine. Although fewer subjects relapsed after concurrent quinine and primaquine therapy than relapsed after chloroquine and primaquine, the difference in cure-rates for Groups 2 and 3 is not statistically significant. The possibility still exists that the dose of chloroquine given in the first 24 hours was insufficient to demonstrate the maximum possible synergistic action of chloroquine with primaquine.

We consider the results to indicate that both of these suppressive agents potentiate the effect of primaquine against late fixed-tissue stages of vivax malaria. The alternate explanation, namely, that primaquine potentiates quinine or chloroquine is considered illogical because no curative action has ever been demonstrated for the latter drugs. The hypothesis that intensification of suppressive action could effect cure was extensively tested during World War II but was abandoned when it was found that a 30-fold increase in the minimum plasma concentration (of chloroquine) necessary to eliminate erythrocytic stages (trophozoites) failed to have demonstrable effect against fixed-tissue stages.[22]

CONCLUSION

Evidence is presented which indicates that either quinine or chloroquine, when administered concurrently with primaquine during the treatment of clinical attacks of mosquito-induced Chesson strain vivax malaria, potentiates the curative action of primaquine.

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THE GASTRIC SECRETORY RESPONSE TO HISTALOG: ONE-HOUR BASAL AND HISTALOG SECRETION IN NORMAL PERSONS AND IN PATIENTS WITH DUODENAL ULCER AND GASTRIC ULCER

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INTRODUCTION

PRELIMINARY observations have indicated that Histalog (3-beta aminoethyl pyrazole), an analog of histamine, stimulates gastric secretion with less tendency to side effects than histamine.[1] The present investigation was undertaken to determine more completely the secretory response to Histalog and to evaluate the clinical tolerance for this compound. In addition, one-hour basal secretion and the one-hour response to Histalog were compared in normal persons and in patients with duodenal ulcer and gastric ulcer.

PROCEDURE AND MATERIAL

The method of investigation has been described.[1, 2] Analyses were performed between 8 and 10 A.M., after an overnight fast. A Rehfuss or Levine tube was introduced into the stomach and the tip was located at the level of the antrum. The residual contents were removed and discarded. Continuous aspiration then was maintained by constant suction, with the patient in the sitting position. Secretions accumulating in the mouth were expectorated. Collections were made in four fifteen-minute periods (one-hour basal). Histalog then was administered subcutaneously in doses of 0.5 mg. per kilogram of body weight and aspirations were continued for a second hour (one-hour Histalog).* The volume of secretion, concentration, and output of hydrochloric acid in each specimen were measured in the usual manner; hourly totals were calculated from these figures.

Data were obtained in 584 normal persons (304 men, 280 women); 231 patients with duodenal ulcer (173 men, 58 women); and 22 patients with gastric ulcer (15 men and 7 women); a total of 837 basal-Histalog analyses. The results were examined by the standard "t" test to quantitate differences between basal and Histalog secretion and to compare statistically the acid output after Histalog with the response to histamine, described previously.[2]

RESULTS

Histalog increased gastric secretion significantly in all patients. Basal secretion in the present study and in the histamine series corresponded very closely, providing a valid basis for comparison of the two sets of data. The output of hydrochloric acid after Histalog exceeded the response to histamine in most patients; for the entire series, the larger responses to Histalog were significant statistically at the 1 per cent probability level. However, the differences are not important clinically, except possibly in the study of suspected achlorhydria when the most potent gastric stimulation is indicated.

From the Department of Medicine, University of Chicago.
Histalog was supplied in generous amounts by Eli Lilly & Company, Indianapolis, Ind.
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*In dogs large doses of Histalog, 3 to 4 mg. per kilogram of body weight, evoke maximal gastric secretory responses within 90 minutes, the effect continuing for several hours.

Side Effects.—Histamine, in doses of 0.01 mg. per kilogram body weight, almost invariably caused an uncomfortable flushing and throbbing sensation in the head. More than 90 per cent of the patients developed headaches; in approximately 50 per cent, the headaches were very severe. Weakness and nausea occurred in 20 per cent; a mild state of shock, with transient unconsciousness, developed in approximately 5 per cent of the patients.

Among more than 2,000 patients* receiving Histalog, a sense of flushing occurred in 20 per cent; only 1 per cent reported actual discomfort. Headaches were described by 3 per cent of the patients, but were pronounced in only two patients. Weakness, nausea, and faintness developed in 1 per cent; and transient syncope in only 0.2 per cent (four patients). These findings indicate that Histalog, in quantities 50 times larger than those of histamine, is approximately 25 times less toxic. In patients receiving both compounds at different times, the preference for Histalog was unanimous.

TABLE I. BASAL-HISTALOG GASTRIC SECRETION IN NORMAL MALES (AVERAGES)

		BASAL			HISTALOG		
			HCl			HCl	
AGE DECADE	NO. OF PERSONS	VOLUME (ML.)	CONC. (C. U.)	OUTPUT (MG.)	VOLUME	CONC.	OUTPUT
20-29	25	73	31	81	167	85	539
30-39	80	80	32	94	167	83	511
40-49	80	83	29	97	171	79	527
50-59	79	69	23	71	142	75	419
60+	40	64	23	63	120	65	316
Total	304						

TABLE II. BASAL-HISTALOG GASTRIC SECRETION IN NORMAL FEMALES (AVERAGES)

		BASAL			HISTALOG		
			HCl			HCl	
AGE DECADE	NO. OF PERSONS	VOLUME (ML.)	CONC. (C. U.)	OUTPUT (MG.)	VOLUME	CONC.	OUTPUT
20-29	22	77	27	79	117	73	316
30-39	66	65	22	58	123	72	316
40-49	80	65	19	46	126	68	329
50-59	80	56	14	36	104	58	255
60+	32	44	15	34	92	54	236
Total	280						

Basal-Histalog Secretion: Group Comparisons.—

Normal (Tables I and II): The volumes of one-hour basal secretion and the concentrations and outputs of hydrochloric acid were higher in normal males than in females, at each decade. In men, the average volume decreased after the age of 50; in women, the average volume diminished steadily with advancing age. Similar trends were noted in the concentration and output of hydrochloric acid. Anacidity throughout the entire basal period was observed in 22 per cent of normal males and in 30 per cent of females. The outputs of hydrochloric acid after Histalog were much higher in normal males than females; however, the proportional increases above basal levels were approximately similar in

*Less than one-half of the 2,000 cases (837) were utilized in the present analysis. The remainder were eliminated for various reasons: a second test, gastric irradiation for peptic ulcer, and the presence of pernicious anemia or gastric cancer.

both sexes. There was a definite correlation between basal and Histalog secretion in the same person. Anacidity after Histalog was noted in 4 per cent of both normal men and women; Histalog anacidity was more frequent after the age of 50 years.

Duodenal ulcer (Tables III and IV): Basal secretion was considerably higher in males with duodenal ulcer than in females, at all age levels. In both sexes, basal secretion greatly exceeded normal values; women with duodenal ulcer secreted more acid under basal conditions than normal men. In males, the output of hydrochloric acid decreased after the age of 60; basal secretion in females with duodenal ulcer did not change appreciably at various decades.

TABLE III. BASAL-HISTALOG GASTRIC SECRETION IN MEN WITH DUODENAL ULCER (AVERAGES)

		BASAL			HISTALOG		
			HCl			HCl	
AGE DECADE	NO. OF PATIENTS	VOLUME (ML.)	CONC. (C. U.)	OUTPUT (MG.)	VOLUME	CONC.	OUTPUT
20-29	10	124	49	208	258	97	906
30-39	40	124	43	202	224	89	728
40-49	61	126	46	220	223	89	720
50-59	39	119	45	211	225	89	753
60+	23	80	48	136	195	99	707
Total	173						

TABLE IV. BASAL-HISTALOG GASTRIC SECRETION IN WOMEN WITH DUODENAL ULCER (AVERAGES)

		BASAL			HISTALOG		
			HCl			HCl	
AGE DECADE	NO. OF PATIENTS	VOLUME (ML.)	CONC. (C. U.)	OUTPUT (MG.)	VOLUME	CONC.	OUTPUT
30-39	10	69	34	100	155	88	489
40-49	27	76	34	99	143	82	442
50-59	13	98	34	117	155	76	414
60+	8	67	38	102	133	85	445
Total	58						

Anacidity throughout all four basal periods was noted in only two women of the 231 patients in this group (1 per cent). The responses to Histalog were proportionately similar in both sexes and the increases above basal levels approximated the normal responses. There was not a single instance of Histalog anacidity in the duodenal ulcer group.

Gastric ulcer (Table V): This group is considered in its entirety because of the small number of cases. The volumes of basal secretion approximated normal values, whereas the concentrations of hydrochloric acid were slightly

TABLE V. BASAL-HISTALOG GASTRIC SECRETION IN GASTRIC ULCER (AVERAGES)

		BASAL			HISTALOG		
			HCl			HCl	
	NO. OF PATIENTS	VOLUME (ML.)	CONC. (C. U.)	OUTPUT (MG.)	VOLUME	CONC.	OUTPUT
Male	15	70	18	51	192	73	522
Female	7	66	22	52	125	64	298
Total	22						

less than normal. Continuous basal anacidity was noted in three patients (14 per cent). The responses to Histalog approximated normal results. Hydrochloric acid was demonstrable after Histalog in all patients with gastric ulcer.

COMMENT

This study confirms preliminary observations indicating that Histalog is a potent gastric secretory stimulant with far less tendency to uncomfortable side effects than histamine. The outputs of hydrochloric acid were larger after Histalog than after histamine, but the differences are not important clinically.

The present data also demonstrate conclusively the significantly higher basal secretion in men and women with duodenal ulcer than is observed in normal persons and in patients with gastric ulcer; the differences are apparent at all age levels. The larger outputs of acid in men, both in the normal and the duodenal groups, also are confirmed. The continuous basal anacidity in 26 per cent of normal persons and 14 per cent of those with gastric ulcers is in contrast to the incidence of 1 per cent in the duodenal ulcer group; the two patients with basal anacidity secreted ample amounts of acid after Histalog. Similar trends differentiating duodenal ulcer from other groups were noted in the frequency distribution of one to four basal periods of anacidity. The outputs of acid after Histalog were largest in patients with duodenal ulcer. However, the Histalog response did not differentiate patients with duodenal ulcer from normals or gastric ulcers as clearly as the 1-hour basal secretion. The data thus re-emphasize the usefulness of the 1-hour basal secretion as an index of the individual patient's gastric secretion, paralleling the more elaborate but less practical measurement of the 12-hour nocturnal gastric secretion.

SUMMARY

The gastric secretory response to Histalog (3-beta aminoethyl pyrazole), an analog of histamine, was measured in 837 patients: 584 with no organic disease, 231 with duodenal ulcer, and 22 with gastric ulcer. In doses 50 times larger, Histalog stimulated higher outputs of hydrochloric acid than histamine, with only minimal side effects. Basal secretion in patients with duodenal ulcer greatly exceeded the output of acid in normal persons and in patients with gastric ulcer, and basal anacidity in this group was rare. Basal secretion in patients with gastric ulcer approximated normal values. The proportional increases above basal levels after Histalog in patients with duodenal ulcer apparently did not exceed the responses of normal persons or patients with gastric ulcer.

CONCLUSIONS

1. Histalog, an analog of histamine, is a very potent stimulant of gastric secretion, with far less tendency to side effects than histamine.

2. The gastric secretory response to Histalog is proportionately similar in patients with duodenal ulcer, normal persons, and those with gastric ulcer, the differences in acid output being determined chiefly by contrasting levels of basal secretion.

3. Basal secretion in patients with duodenal ulcer is much higher than normal, and continuous basal anacidity in this group is very rare.

4. The two-hour combined basal-Histalog test is a relatively simple, yet comprehensive and clinically useful measure of the individual gastric secretory pattern.

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LABORATORY METHODS

AN EFFICIENT CLINICAL METHOD FOR DETERMINING THE CO_2 CONTENT OF SERUM

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INTRODUCTION

THE CO_2 content of a serum sample can be determined simply and rapidly, using a suitable pH meter. The pH is measured on the serum obtained anaerobically from the patient's blood stream, and again after the sample has been equilibrated with a gas mixture containing a known content of CO_2. From the change in pH, the CO_2 content can be calculated or, more conveniently, read from a nomogram. The entire procedure can be performed in about 2½ minutes and requires only 1 ml. of serum.

PRINCIPLES

The method which has been devised here is based upon the several well-established biochemical laws which govern acid-base equilibriums in serum and plasma. As will be shown, the principal variants bearing on serum pH are the partial pressure of CO_2, the concentration of fixed acids and bases, and the net concentration of the various buffers in the serum. Under the conditions of this procedure, that is, the equilibration of the serum with gas of a known partial pressure of CO_2 (pCO_2), the net concentration of fixed acids, bases, and serum buffers is uninfluenced. Knowing the pH at the established pCO_2 the net concentration of these serum buffers, which have been termed "the buffer base,"[1] can be determined, and knowing this value and the original pH of the serum, the CO_2 content of the serum as drawn from the patient can be readily calculated.

An equation can be derived expressing the total CO_2 content in terms of the two pH values and the pCO_2 of the gas mixture. The calculations are based on the classical equations expressing the interrelationships of the various parameters of the pH-CO_2 system:

$$pH = 6.1 + \log \frac{(HCO_3^-)}{(H_2CO_3)} \quad \text{(Ref. 2)} \qquad \text{where } 6.1 = pK^1 \quad \text{(Ref. 3)} \qquad (1)$$

$$(CO_2) = (H_2CO_3) + (HCO_3^-) \quad \text{(Ref. 2)} \qquad (2)$$

$$(H_2CO_3) = .0591\ \alpha\ pCO_2 \text{ where } \alpha = \text{the solubility coefficient} = .510 \quad \text{(Ref. 4)}$$

$$(H_2CO_3) = .03\ pCO_2 \qquad (3)$$

It has been shown that changing the pCO_2 of serum changes the hydrogen ion concentration (H^+) in the following fashion,

$$pCO_2 \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$$
$$\downarrow\uparrow$$
$$H^+ + Pr^- \rightleftarrows HPr \quad \text{(Ref. 2)}$$

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where Pr^- is the plasma protein able to buffer the H^+ ions. Thus, for example, a decrease in the pCO_2 will result in a shift in the equilibrium so that a certain quantity of HCO_3^- will form H_2CO_3 and be lost as CO_2; but an equal amount of HPr must ionize to Pr^- to supply the hydrogen ions necessary to form the H_2CO_3. Although such shifts in pCO_2 affect the concentration of the individual parameters, the sum of the concentrations of Pr^- and HCO_3^- remains constant. It is this sum which has been defined as "the buffer base." Its concentration can be changed only by the addition of fixed acid or base, but once the serum is outside the body this does not occur. Thus, under the conditions of this procedure, the buffer base remains constant.

$$\text{Buffer base} = (K) = (HCO_3^-) + (Pr^-) \quad (4)$$

The concentration of negative charges in the plasma proteins capable of accepting H^+ ions (Pr^-) is 5.4 (pH – 4.8) in separated serum where 5.4 represents the average buffer value of the serum proteins and 4.8 is the isoelectric pH at which no net negative charges exist on the proteins.[5] Substituting this value for Pr^- in equation (4),

$$(K) = (HCO_3^-) + 5.4\ (pH - 4.8) \quad (5)$$

In this procedure, the buffer base (K) is first determined on the equilibrated serum and must be expressed in terms of the pH of that serum (pH_2) and the pCO_2 of the equilibrating gas mixture. The expression for K is derived as follows:

$$pH_2 = 6.1 + \log \frac{(HCO_3^-)}{(H_2CO_3)}$$ Substituting from (3),

$$pH_2 = 6.1 + \log \frac{(HCO_3^-)}{.03pCO_2}$$ Solving,

$$(HCO_3^-) = .03pCO_2 \times \text{antilog}\ (pH_2 - 6.1)$$ Substituting in (5),

$$(K) = .03pCO_2 \times \text{antilog}\ (pH_2 - 6.1) + 5.4\ (pH_2 - 4.8). \quad (6)$$

The total CO_2 content of the original serum can be expressed in terms of the pH of that serum (pH_1) and the buffer base (K) as calculated from equation (6).

$$pH_1 = 6.1 + \log \frac{(HCO_3^-)}{(H_2CO_3)}$$ Substituting from (2),

$$pH_1 = 6.1 + \log \frac{(HCO_3^-)}{(CO_2) - (HCO_3^-)}$$ Solving,

$$(CO_2) = \frac{1 + \text{antilog}\ (pH_1 - 6.1)}{\text{antilog}\ (pH_1 - 6.1)} \times (HCO_3^-)$$ Substituting from (5),

$$(CO_2) = \frac{1 + \text{antilog}\ (pH_1 - 6.1)}{\text{antilog}\ (pH_1 - 6.1)} \times [(K) - 5.4\ (pH_1 - 4.8)]$$

Substituting from (6) and simplifying,

$$(CO_2) = \frac{1 + \text{antilog}\ (pH_1 - 6.1)}{\text{antilog}\ (pH_1 - 6.1)} \times [.03pCO_2 \times \text{antilog}\ (pH_2 - 6.1) + 5.4\ (pH_2 - pH_1)]$$

The total CO_2 content can be more easily determined from the same three parameters using a nomogram (Fig. 1). If a ruler is positioned to intersect

the pH_2 value on the pH scale and the pCO_2 of the gas mixture on the pCO_2 scale, it intersects a point on the pivot line which represents the buffer base concentration in the sample.[1] The ruler may then be pivoted around the latter point until it intersects the pH_1 value on the pH scale. It will then intersect the correct values for all the parameters in the serum before equilibration. The total CO_2 content can then be read directly from the nomogram.

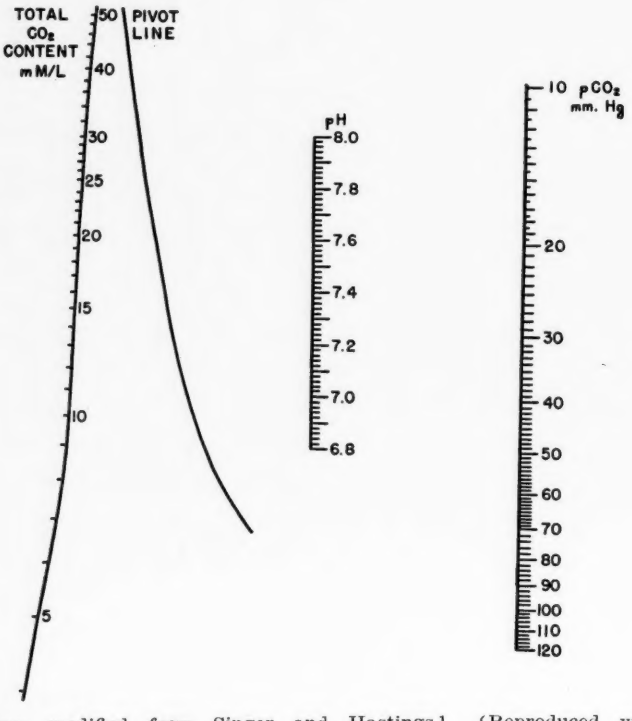

Fig. 1.—Nomogram modified from Singer and Hastings.[1] (Reproduced with permission of Dr. Richard B. Singer.)

MATERIALS

1. A pH meter which is capable of measuring the pH under anaerobic conditions to an accuracy of ± 0.01 pH unit was used. The electrode chamber should be held at a constant temperature of 37° C. and should be shaped so that gas can be bubbled through the sample (Fig. 2).

2. A tank was used which contained a gas mixture of about 5 to 6 per cent CO_2 and 94 to 95 per cent N_2. The exact composition of CO_2 to three significant figures can be determined using a Haldane or van Slyke manometric apparatus. A large tank lasts a year or more under normal laboratory use and needs to be standardized only once during that time. The pCO_2 of the gas can be calculated using the equation:

$$pCO_2 = \frac{\%CO_2 \text{ in tank}}{100} \times (\text{barometric pressure} - 50) \qquad \text{(Ref. 2)}$$

where per cent of CO_2 is expressed as the dry gas, the barometric pressure is expressed in mm. Hg, and 50 represents the vapor pressure of H_2O at 37° C. If the average of the barometric pressure in the vicinity is determined and used constantly, daily calculations will

not be necessary. In Chicago, it was found that daily fluctuations of the barometric pressure caused an error not greater than ±1 meq. per liter of CO_2 and usually less than ±0.3 meq. per liter when the average barometric pressure was used. The data presented in Table 1 were determined using such an average value.

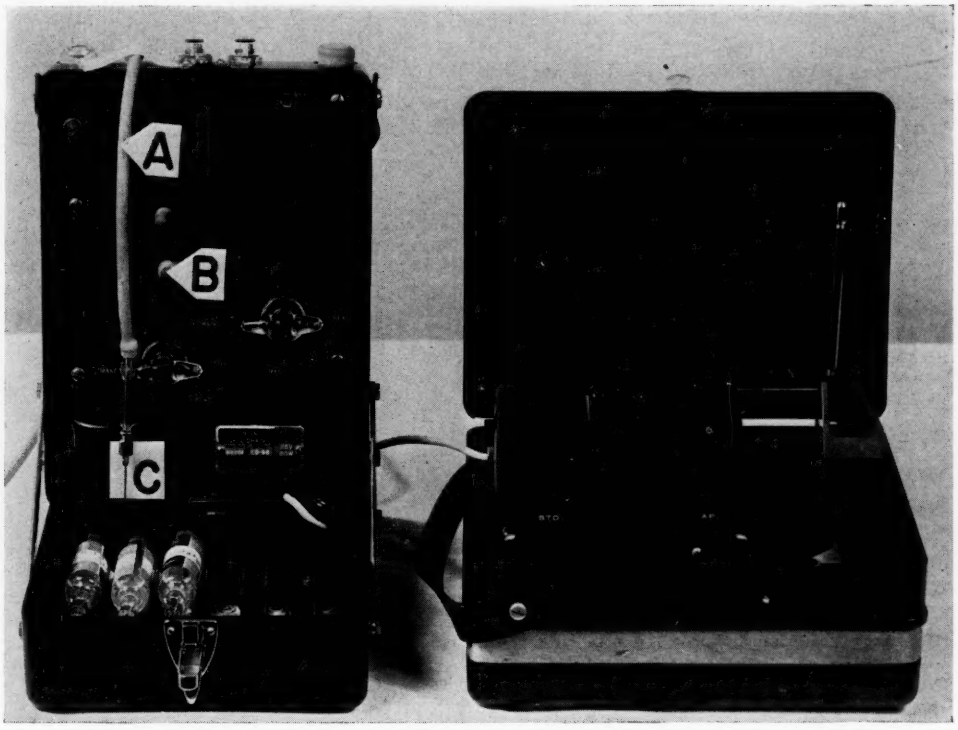

Fig. 2.—This pH meter houses a clinical cell chain capable of measuring pH anaerobically at 37° C. and has a sample chamber of convenient design. *A*, Gas delivery tube; *B*, sample chamber; *C*, gas delivery needle. (Manufactured by and reproduced with the permission of the Coleman Instrument Company, Maywood, Illinois.)

3. A gas delivery needle and narrow bore rubber tubing were assembled as shown in Fig. 2. The needle is a 30-gauge 1-inch hypodermic needle. A 20-gauge 1-inch needle with a copper sinter in its tip effects equilibration more rapidly but is difficult to make.

PROCEDURE

Collect the blood anaerobically under a layer of mineral oil, centrifuge, and collect serum anaerobically. If the serum has been kept in a refrigerator allow it to warm to room temperature before proceeding with the determinations. Allow the gas to flow slowly (50 c.c. per minute) through the tubing and out the delivery needle throughout the period during which determinations are to be made. Wash the sample chamber of the pH meter with a 10/1 million dilution of antifoam AF* between each determination. Inject about 1 c.c. of serum into the sample chamber of the pH meter and determine the pH (pH_1). Then place the delivery needle in the sample chamber and allow the gas to bubble through the serum until the maximum pH change has developed (about 1.5 minutes with a 30-gauge 1-inch delivery needle). Read the pH again (pH_2), and determine the total CO_2 content from the nomogram as follows:

1. Place a ruler so that it intersects the pH_2 value on the pH scale and the pCO_2 of the gas mixture on the pCO_2 scale.

*Dow Corning.

2. Place tip of pencil on the pivot line at the point where the ruler intersects it.
3. Pivot ruler around the pencil tip until the ruler intersects pH_1 reading on the pH scale. The ruler now intersects the correct CO_2 content on the appropriate scale.

RESULTS

The values obtained by this method were compared with those obtained on a van Slyke manometric apparatus. The results are noted in Table 1.

TABLE I

SAMPLE NO.	pH	CO_2 CONTENT (THIS METHOD)	CO_2 CONTENT (VAN SLYKE)
1	7.50	28.8	29.8
2	7.44	31.0	31.9
3	7.42	30.5	30.4
4	7.40	28.6	28.7
5	7.54	32.6	31.0
6	7.28	14.7	14.7
7	7.29	20 4	21.9
8	7.43	20.0	20.7
9	7.39	25.4	26.5
10	7.32	16.8	17.2
11	7.36	16.4	16.3
12	7.32	20.8	20.1
13	7.52	33.4	35.0
14	7.30	33.8	35.2
15	7.50	21.7	20.5
16	7.43	32.6	34.1
17	7.38	18.0	18.0
18	7.45	28.2	28.3
19	7.39	20.6	19.0
20	7.38	21.4	21.3
21	7.52	35.7	37.0
22	7.38	26.0	26.7
23	7.34	22.4	23.8
24	7.41	26.8	28.4
25	7.47	29.1	30.2
26	7.29	15.8	17.0
27	7.32	26.6	26.0
28	7.57	38.9	39.8
29	7.43	29.1	30.8
30	7.44	32.5	33.7
31	7.42	26.4	26.4
32	7.34	28.0	28.6
33	7.36	23.7	23.8
34	7.41	20.0	19.9
35	7.44	23.5	23.5
36	7.42	29.5	29.6
37	7.33	23.8	23.2
38	7.34	20.6	21.0
39	7.35	29.5	30.4
40	7.50	31.3	31.0
41	7.46	31.8	32.7
42	7.45	28.0	26.9
43	7.43	24.7	23.0
44	7.28	25.5	25.4
45	7.46	9.8	10.2
46	7.35	24.9	24.7
47	7.39	14.2	14.0
48	7.54	8.2	8.5
49	7.32	14.0	14.4
50	7.49	21.7	22.4

The standard deviation is 0.92 meq. per liter of CO_2.

SUMMARY

The total CO_2 content of a serum sample can be determined quickly and accurately using a suitable pH meter. The pH of the serum is determined before and after equilibration with a gas containing a known percentage of CO_2. From the change in pH, the CO_2 content of the sample can be calculated or read from a simple nomogram.

I am indebted to Dr. Richard L. Landau, Dr. John F. Perkins, Jr., and Miss Ruth Loughead for their valuable counsel and assistance.

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THE USE OF LYOPHILIZED MILK IN THE ASSAY OF URORENNIN

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IN RECENT years there has been an increasing interest in the peptic activity of urine. The present state of our knowledge of this subject and an evaluation of the clinical applications of measurements of the activity are the subjects of an excellent review by D. C. Balfour, Jr.[1]

West, Ellis, and Scott[2] in 1952 described a method of measuring uropepsin by determining the rennin activity of acidified urine. Their method is an adaptation of a procedure described by Sylvest[3] a few years earlier. Sylvest, however, termed the enzyme he was assaying "urorenninogen." Goodman, Sandoval, and Halstead[4] agreed that the method of measuring rennin activity of urine described by West was simple and inexpensive. They pointed out that the substrate used by West, homogenized milk, varied from time to time and from place to place, and that the enzyme activity of the same urine specimen varied, depending on which batch of milk was used as substrate. To overcome this difficulty, they described the preparation of an artificial milk which gave consistently reproducible results. Goodman and his co-workers also suggested that the enzyme activity be reported in terms of micrograms of crystalline pepsin rather than the arbitrarily defined units used by West.

Using standard solutions of crystalline pepsin, we have been able to confirm the variations in activity which are obtained when homogenized milk from different dairies, or even the same dairy on different days, is used (Table I). Unfortunately, in our hands the casein preparation described by Goodman and associates has also not given completely consistent results (Table II). Moreover, while homogenized milk and Goodman's casein solution usually are stable for 10 to 14 days, particularly if Merthiolate has been added, an occasional milk or casein solution substrate will go "off" after a few days. We found that it was necessary to check our substrates every few days with standard pepsin solutions to be sure that they were still reliable.

The variability in homogenized milk and casein solution substrates and the erratic stability necessitate standardization of each batch of substrate when it is prepared, and every few days thereafter if accurate results are desired.

Recently we have found that this necessity for repeated standardization of the substrate can be eliminated by utilizing lyophilized skimmed milk as the substrate for the West assay method.

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TABLE I. VARIATIONS IN REACTION TIME WITH DIFFERENT HOMOGENIZED MILK SUBSTRATES

		SECONDS TO END POINT						
		MICROGRAMS CRYSTALLINE PEPSIN						
DAIRY	SAMPLE	0.5	0.4	0.3	0.2	0.15	0.7	0.05
I.	1	34		52	75		122	
	2	35		51	74		122	205
	3			35	50		85	
	4			45	60	74		
	5			48	64	80		
	6			54	74	93		
	7	45		65	90			
	8			65	90			
II.	1	35		52	73		115	
	2			65	90	114		
	3	45		65	90			
	4			65	90			
	5	48		70	95			
	6	50		73	102			
III.	1			62	35			
IV.	1			63	87			
	2			65	90			
	3			70	93			
	4			65	88			

Preparation of Substrate and Technique of Assay.—Homogenized skimmed milk was shell frozen and dried in lyophilizing apparatus.* The powder which results from this process, lyophilized milk, is readily soluble in water. This powder is stored in stoppered tubes in a desiccator, although the latter precaution is probably not necessary. The stock substrate solution is made by adding 1 ml. of water to each 20 mg. of lyophilized milk. The powder goes into solution readily and the resultant solution suspension is stable for at least one week if kept in the refrigerator. For most hospitals, 5 ml. of water added to 100 mg. of lyophilized milk should afford an ample supply of substrate for the week's work. The substrate actually used in the assay is made up of equal parts of the stock substrate and an acetate buffer of pH 4.9 (NaOH 4.2 Gm. and glacial acetic acid 9.2 ml., distilled water to 100 ml.). The resultant buffered suspension is not stable for more than 24 hours and must be made up on the day the assay is performed.

TABLE II. VARIATIONS IN REACTION TIME WITH TWELVE DIFFERENT BATCHES OF CASEIN SOLUTION SUBSTRATES*

	SECONDS TO END POINT						
	MICROGRAMS CRYSTALLINE PEPSIN						
SUBSTRATE	0.5	0.3	0.25	0.2	0.15	0.1	0.05
1	35		55			105	155
2	35		55			105	160
3	35		53			100	145
4	30		45			80	135
5	35	44		60		95	155
6	36	47		64		95	
7	32	43		52		80	
8	35	47		63		95	
9		80		100	123		
10	35	47		60		93	
11	40	54		71		105	
12		80		100	123		

*Prepared according to directions of Goodman, Sandoval, and Halstead.[4]

*Greiner Glass Blowing Laboratory, 255 West Santa Barbara Avenue, Los Angeles, Calif.

TABLE III. VARIATIONS IN REACTION TIME WITH "BUFFERED LYOPHILIZED MILK" SUBSTRATE AT VARYING INTERVALS AFTER PREPARATION OF THE LYOPHILIZED MILK

DAYS FROM PREPARATION OF SUBSTRATE	SAMPLE	SECONDS TO END POINT				
		MICROGRAMS CRYSTALLINE PEPSIN				
		0.5	0.3	0.2	0.15	0.1
1	1	47	70			
2	2	47	69			
7	3		70	95		
9	4		69	95		
10	5		70			
15	6		69			
72	7	46	69	94		
98	8	46	68	94	121	
105	9	46	68	94		
290	10	46	69	94		

The technique of assay is performed as stated by West, Ellis, and Scott,[2] except that 0.5 ml. of the "buffered lyophilized milk substrate" described previously is used instead of 0.5 ml. of homogenized milk. The end point is found most easily by shaking the tube in front of a black square containing a central aperture 1 cm. in diameter, which is suspended in front of a 60 watt lamp bulb. The contents of the tube are held somewhat below the level of the aperture and the end point is read when precipitate appears and descends in the film on the side of the tube when the tube is shaken. In our experience the determinations are most reliable when the end point is between 50 and 150 seconds.

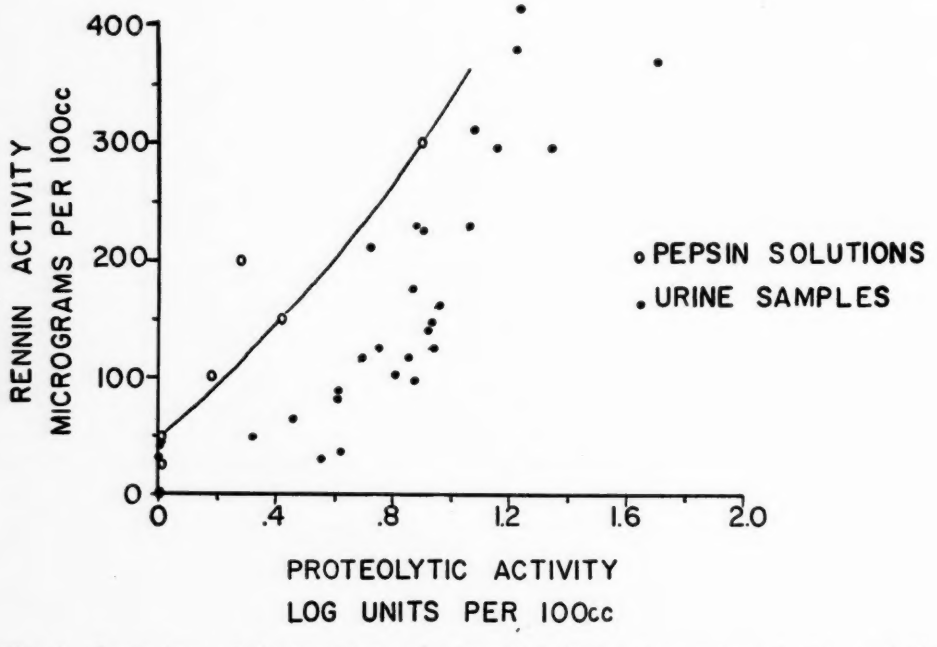

Fig. 1.—Comparison of "proteolytic" and "rennet" activity of a series of (1) crystalline pepsin solutions and (2) urine specimens.

The first "buffered lyophilized milk" substrate prepared is standardized against crystalline pepsin solutions containing 0.5, 0.3, 0.2, 0.15, and 0.1 μg per milliliter. A curve is plotted relating micrograms pepsin to the seconds until the end point is reached. In

subsequent assays, the pepsin activity is read off of this curve. It is not necessary to restandardize substrates made up from the same batch of lyophilized milk, since such substrates give consistently reproducible results for at least three and one-half months (Table III).

The Relationship of Urorenninogen to Uropepsinogen in Human Urine.—The enzyme measured by the rennet method of West and associates, and that determined by variations of the classic proteolytic method of Anson and Mirsky[5] is generally collectively designated as uropepsin. In the present experiments, the rennet and proteolytic activity of a number of urine specimens and of a series of solutions of crystalline pepsin have been determined (Fig. 1). It is apparent that the two activities parallel one another, but urine with a given rennet activity has much more proteolytic activity than does a purified pepsin solution having comparable rennet activity. Possibly the proteolytic assays are measuring enzymes other than pepsinogen. While no clinical error will result from referring to the activity determined by both methods as uropepsin, it would seem more precise to refer to the enzyme assayed by a proteolytic method as uropepsin and that activity measured by a rennet method as urorennin.

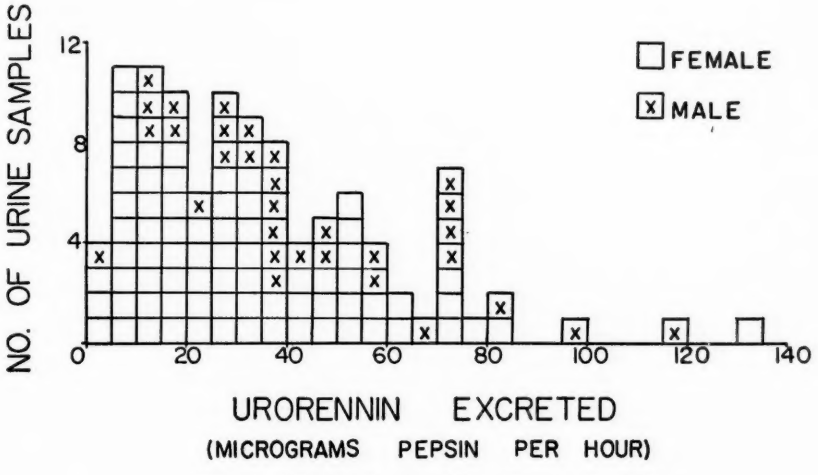

Fig. 2.—Urorennin excretion of normal adults.

Normal Range and Constancy of Urorennin Excretion.—The amount of urorennin excreted in the voided urine from 78 healthy young men and women during one hour was determined. The results are presented in Fig. 2.

The rates of excretion of urorennin by seven young women during periods of one to four hours were determined daily. The same individual showed considerable variation in the amount of urorennin excreted during these short periods of time each day. The variations in excretion of urorennin in one subject compared to variations in excretion of diastase in the same subject are depicted in Fig. 3. The causes of these variations are unknown.

Four individuals having pernicious anemia were found to excrete less than 5 μg of urorennin per hour. A preliminary report of urorennin excretion in patients with duodenal ulcer has been published.[6]

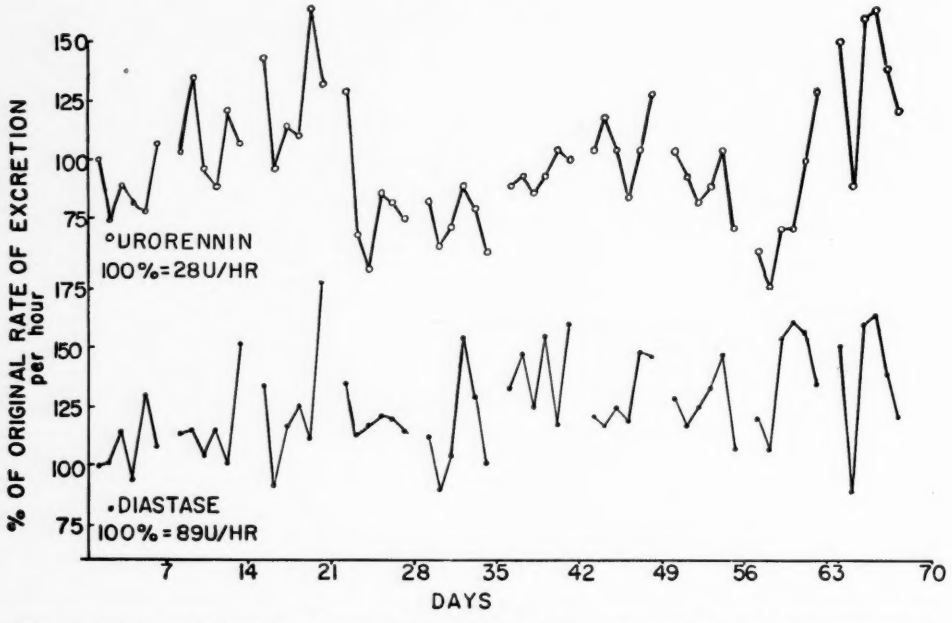

Fig. 3.—Daily variations in amount of urorennin and diastase excreted in a one-hour period.

CONCLUSIONS

1. The use of lyophilized milk as a stable, reproducible substrate for the determination of the rennin activity of urine is described.

2. The "rennin" and "proteolytic" activities of urine specimens and pepsin solutions were compared.

3. The average rate of urorennin excretion in 78 healthy young adults was 39 μg of pepsin per hour.

4. Four patients with pernicious anemia had urorennin excretion rates of less than 5 μg of pepsin per hour.

We are indebted to Dr. A. Mendelhoff for the proteolytic assays which were made by a modification of the procedure of Anson and Mirsky.

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THE DEMONSTRATION OF INHIBITION OF URINARY FORMALDEHYDE PRODUCTION AFTER PERIODIC ACID OXIDATION AS RELATED TO THE VOLUME OF THE URINE SAMPLES OXIDIZED

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ONE of the several methods available for the assay of urinary corticoids is dependent upon measurement of the formaldehyde liberated after oxidation with periodic acid. This method, in common usage in many laboratories, has been criticized because of certain variations in the assays obtained. Hollander and associates,[1] Paterson and Marrian,[2] Mason[3] reported incomplete recovery of added known amounts of formaldehydogenic steroid to urine extracts. Hollander and co-workers[1] suggested that some type of inhibition was present during the course of oxidation, resulting in decreased formaldehyde values. Paterson and Marrian[2] have preferred to explain the decreased values on the basis of retention of the liberated formaldehyde by some unknown substance in the urine. They studied this by measuring the recovery of formaldehyde added to urine extract. Regardless of whether there was "inhibition" of the oxidative process or "retention" of the formaldehyde during distillation, alkaline and aqueous washing of the urine extracts partly eliminated the problem.

It seemed reasonable that if inhibitory substances were present in urine extracts, then whole urine would contain even greater quantities. Utilization of whole urine, therefore, would more readily demonstrate such inhibition despite the fact that it is well known that numerous substances in whole urine other than corticoids liberate formaldehyde after oxidation with periodic acid.

This report deals with formaldehyde values obtained after periodic acid oxidation of untreated whole urine and of chloroform extract of urine. The character of the "inhibitory substances" based upon formaldehyde liberation is discussed.

METHODS

Normal dog urine was collected without added preservative and stored in the frozen state. One-tenth to ten milliliter samples were utilized for oxidation. Distilled water was added so that the total volume of each sample was equal to that of the largest sample being tested. Addition of water, within the range employed, did not alter the result of the oxidation. Oxidation was carried out by the addition of 2 ml. of 0.05 M periodic acid in 0.4 M sulfuric acid to the respective urine samples. The volume of periodic acid was increased in a number of experiments. Forty-five minutes was allowed for completion of oxidation. This time interval was changed when the duration of oxidation was designed

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as the variable. Oxidation was arrested by the addition of 1.5 ml. of 14 per cent stannous chloride in N HCl solution. In the experiments in which the volume of the periodic acid was altered, there was a corresponding adjustment in the volume of stannous chloride solution. The pH values of the oxidation mixture were higher in larger urine samples than in smaller ones. Adjustment of the pH to equally low values in each sample did not alter the result within the experimental error. Distillation and colorimetric estimation followed oxidation. A modification of the method of Daughaday and associates was used.[4]

Distillation.—The reaction mixture was transferred to a microdistilling flask, rinsed with 3 ml. of 5 M sulfuric acid. The volume was adjusted with water to a total of 19 ml.; 14 ml. of distillate was collected in 2 ml. of a 4 per cent sodium sulfite solution containing 1 per cent alcohol. The last milliliter of the distillate rinsed the inside of the condenser; the outer surface of the tip was rinsed with 1 ml. of water.

Colorimetry.—To determine the formaldehyde concentration in the distillate, 5 ml. of 0.2 per cent chromotropic acid in 15 M sulfuric acid was added to 3 ml. of the properly diluted unknown solution. After heating in boiling water for 30 minutes, the intensity of the color was read in a Coleman Jr. spectrophotometer at 570 mμ. (1) The reference blank was composed of water and color reagent. (2) Blanks composed of water treated as the urine samples through the oxidation procedure gave identical readings with the reference blanks. (3) Unoxidized urine blanks interfered slightly only if large volumes of urine were used. The interference observed was due to a yellow color instead of the clear purple obtained with the formaldehyde color reaction of the oxidized urine.

Recovery of Added Formaldehyde.—Known amounts, 0.5 to 3 ml. of a 3.4 mg. per cent hexamethylenetetramine solution were added to the oxidized and unoxidized urine samples before distillation.

Formaldehyde standards were prepared by distillation of a known amount of hexamethylenetetramine solution in the presence of sulfuric acid. Color development followed as described previously. Calculation was based upon formaldehyde concentration expressed in milligrams per cent or in micrograms per individual oxidized aliquot.

Urine extracts were also oxidized. Acidified urine samples were extracted three times with equal volumes of redistilled chloroform. Washed and unwashed aliquots of the extract were oxidized in the usual manner. The washing of extracts was done with 0.1 N sodium hydroxide and water. The extract was then dried over sodium sulfate, evaporated at low temperature and the residue taken up in acetic acid.

RESULTS

It is shown in Fig. 1 that the concentration of formaldehyde liberated decreases as the volume of urine oxidized is increased. This decrease is approximately proportional to the increase of volume. Consequently, the individual oxidized values show a simple regularity.*

Experiments performed on urine extracts as well as whole urine also demonstrated that the formaldehyde concentrations decreased as the volumes of the urine samples were increased. Washed versus unwashed extracts were compared. It is shown in Fig. 3 that the formaldehyde values of washed extract did not decrease or decreased only slightly.

Oxidation of whole urine was carried out with reference both to amounts of periodic acid employed and the time oxidation was allowed to continue.

*That is, each value is smaller than the theoretical value calculated from the curve of the per cent obtained by extrapolating to zero volume. The greater the oxidized volume, the greater is the difference between the theoretical value and the experimental value. These differences in values, measured in a series of evenly increasing volumes, increase in a second degree arithmetic progression (1^2A, 2^2A, 3^2A, and so on; A=the initial difference) (Fig. 2).

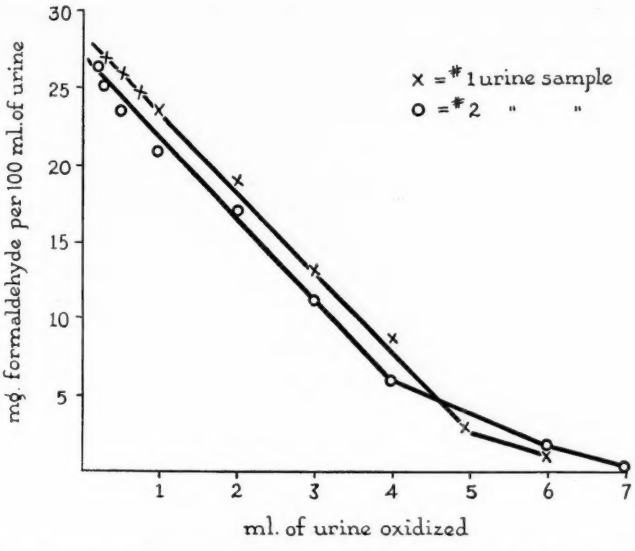

Fig. 1.—The decrease of the concentration of formaldehyde after oxidation of urine as the amounts of the urine samples were increased.

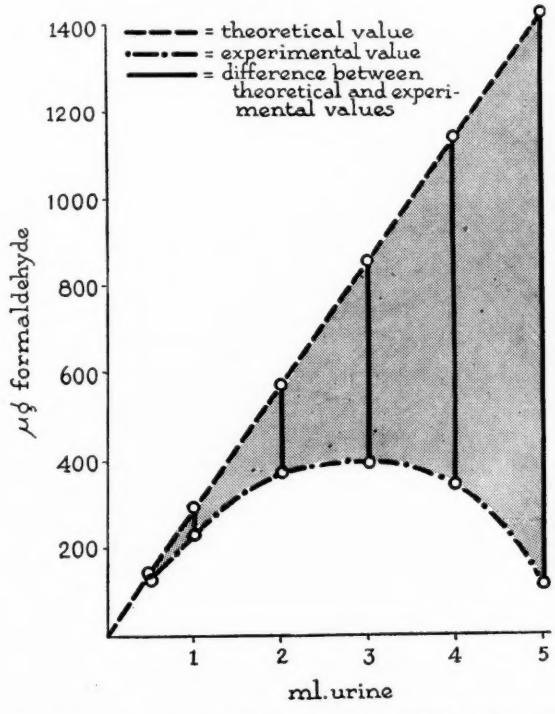

Fig. 2.—Analysis of the differences between the theoretical values and the experimental values obtained after oxidation of varying volumes of whole urine.

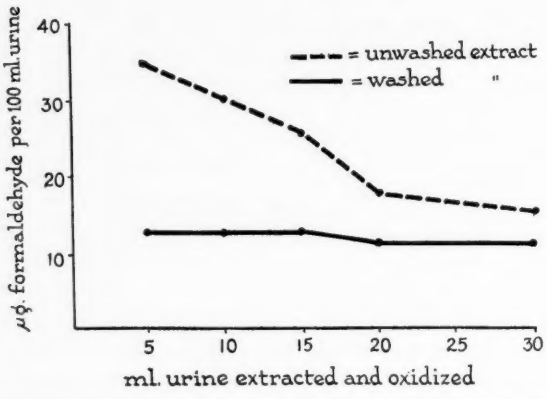

Fig. 3.—Washed urine extract decreased to a lesser degree and began to decrease at a larger volume than unwashed extract.

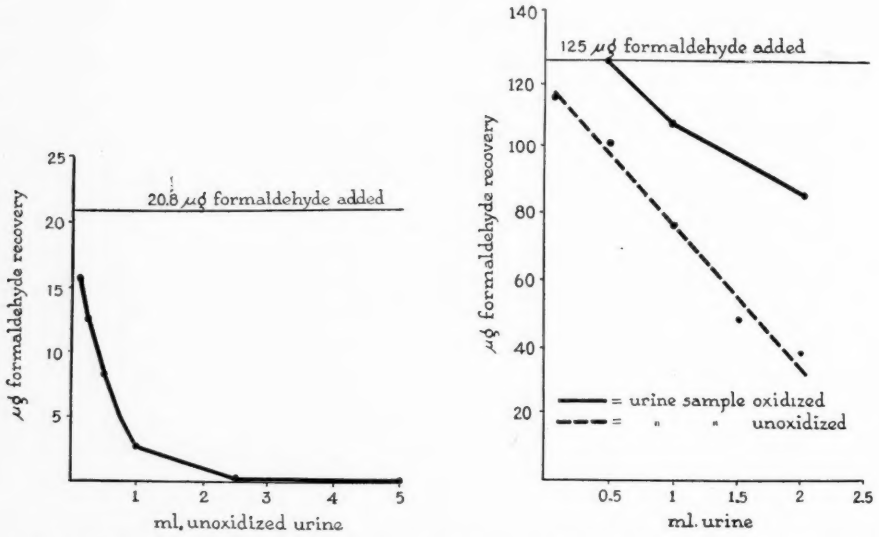

Fig. 4.—Recovery of a constant amount of formaldehyde added to different volumes of unoxidized urine samples.

Fig. 5.—Oxidized urine sample retained the added formaldehyde to a lesser degree than unoxidized urine sample.

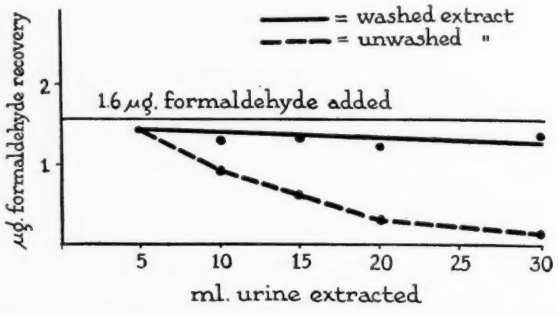

Fig. 6.—Recovery of formaldehyde added to various volumes of washed and unwashed urine extract.

Formaldehyde liberation was constant despite variations of from 2 to 4 ml. of periodic acid and from 45 to 90 minutes for durations of oxidation, within limits of the urine volumes employed.

Experiments with formaldehyde added to a series of increasing volumes of whole urine resulted in incomplete recovery of the added formaldehyde both in the urine sample which was oxidized and in that which was unoxidized. In one instance, 0.1 ml. sample of unoxidized urine retained 5 μg of the added formaldehyde (Fig. 4). Increased volumes of urine resulted in increased formaldehyde retention. It is shown in Fig. 5 that the oxidized urine samples retained the added formaldehyde to a lesser degree than similar aliquots of unoxidized urine samples.

The recovery of added formaldehyde was nearly complete when a constant amount of exogenous formaldehyde was added to various volumes of washed urine extract (Fig. 6). Recovery of the same amount of formaldehyde, however, decreased sharply when unwashed aliquots were used in the same volume series.

DISCUSSION

The decrease in endogenous formaldehyde values obtained with increased volumes of oxidized urine indicates that there is inhibition of the production of formaldehyde.* It is suggested that inhibition can be demonstrated better by variation of the volume of urine than by recovery of added formaldehydogenic steroid. This proposed variation of volume offers a dynamic approach to the problem rather than the static one of an arbitrarily chosen constant volume.

Observation by this approach confirmed Paterson and Marrian's finding[2] that washing the urine extract resulted in more complete recovery of added formaldehyde, indicating decreased inhibition of the formaldehyde values.

Disagreement exists[1, 2] as to whether diminished formaldehyde production is caused by: (1) a true inhibitor of the oxidative procedure—if this be the case, then increasing periodic acid or the time of oxidation should result in increased formaldehyde values when constant volumes of urine are used; or (2) retention of the liberated formaldehyde—if this were occurring, then increasing the periodic acid or the time of oxidation should not result in increasing formaldehyde values. Retention occurs after and independently of the oxidative procedure.

Failure to demonstrate any alteration in the amounts of formaldehyde liberated within the range of the volumes of urine employed, where time of oxidation and volume of oxidizing agent were each the variable factor, clearly indicated that no inhibition of the oxidative process could be demonstrated in these experiments.

The recovery of formaldehyde added to oxidized and unoxidized urine samples was found to be a direct function of the volume of the urine sample being tested. The absence of endogenous formaldehyde in unoxidized urine

*Wilson[5] noticed changed values when she oxidized extracts of different volumes of urine.

samples caused even greater retention of the added formaldehyde. This suggests that formaldehyde-retaining substances are present in the urine and are a function of the urine volumes being assayed.

Heat lability and consequent damage during distillation of the chloroform extract, as suggested by one group,[6] is not relevant to these observations, as oxidation was the first step, and manipulation with chloroform extract was absent altogether in our experiments with whole urine.

SUMMARY

Urine extract and whole urine were oxidized with periodic acid and the liberated formaldehyde determined. Varying values of formaldehyde were obtained when a series of different volumes of urine were oxidized. Oxidation of increased volumes of urine resulted in correspondingly decreased formaldehyde concentration. The presence of "formaldehyde-retention" versus "inhibition of oxidation" is discussed.

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PENICILLIN BLOOD AGAR MEDIUM FOR USE IN ROUTINE DIAGNOSTIC CULTIVATION OF MYCOBACTERIUM TUBERCULOSIS

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INTRODUCTION

THE practicability of using various types of blood media for the cultivation of *Mycobacterium tuberculosis* has been proved for some time.[1-4] Experiments in our laboratory as well as elsewhere[5-8] show that blood media are valuable when used for the cultivation of tubercle bacilli from routinely submitted material of patients with clinical tuberculosis. When penicillin is added to these blood media, contamination can be kept at a minimum. The study presented here compares the results obtained on penicillin blood agar medium, plain blood agar medium, and Lowenstein-Jensen medium used under routine diagnostic conditions.

METHODS

All specimens were cultured on three media: blood agar, penicillin blood agar, and Lowenstein-Jensen medium. The blood agar was made according to Frisch and Tarshis[2] as follows: blood agar base (Difco) 3.0 Gm., glycerin (analytical grade) 1.0 ml., distilled water 74.0 ml., banked human blood 25.0 ml. with standard acid citrate and dextrose added. The blood agar base was hydrated and dissolved by heating. Glycerin was added and the solution was sterilized in the autoclave for 15 minutes at 15 pounds pressure. After cooling to 55° C., 3- to 4-week-old blood was added aseptically. The medium was then poured into sterile screw-cap tubes and slanted. After incubation at 37° C. for 48 hours, to test sterility, it was stored. The penicillin blood agar was made in the same manner as plain blood agar, but 750 units of penicillin G (Pfizer) in 1 ml. of distilled water was added to the medium after the agar was cooled to 55° C. and before the blood was added. The concentration of penicillin used was 75 units per milliliter of medium.[2] The Lowenstein-Jensen medium was purchased from the Difco Laboratories.

The specimens used for culturing included either morning or 24-hour sputa, gastric washings, and material from bronchoscopic examinations, from patients who either were not treated at all with chemotherapy or were receiving streptomycin and para-aminosalicylic acid or isoniazid or viomycin or a combination of these drugs. The sodium hydroxide concentration method was used and one tube of each of the three media was inoculated. All tubes were incubated at 37° C. in a slanted position for one week and then in the upright position. The cultures were examined daily under a bright light and those which were negative were kept for 8 weeks before they were reported as such. Smears were made from colonies which appeared questionable on gross examination and these were stained and examined microscopically. Cultures positive on blood media only were confirmed by guinea pig inoculation.

RESULTS

As can be seen from a perusal of Tables I to IV, the incidence of growth was highest on penicillin blood agar with 94.6 per cent of the total 260 positive

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cultures, 90 per cent on Lowenstein-Jensen, and 86.9 per cent on blood agar (Table II). Contamination (Table III) was highest on blood agar with 9.3 per cent, 2.4 per cent on penicillin blood agar, and 0.7 per cent on Lowenstein-Jensen medium. The time of first appearance of growth was 18.2 days on penicillin blood agar, 18.5 days on blood agar, and 18.7 days on Lowenstein-Jensen medium.

In Table V an attempt is made to summarize the results of the experiments in the literature which have used methods similar to ours or approaching our own.

TABLE I. GENERAL ANALYSIS OF SPECIMENS

Total number of specimens	710
Total number of positives	260
Total number of negatives	550
Total number of contaminated	88

TABLE II. COMPARISON OF GROWTH INCIDENCE ON DIFFERENT MEDIA

	NUMBER	PER CENT
Positive on penicillin blood agar	246	94.6
Positive on blood agar	226	86.9
Positive on Lowenstein-Jensen	234	90.0
Positive on all three media	206	79.2
Total number of positive cultures	260	100.0

TABLE III. COMPARISON OF CONTAMINATION

	NUMBER	PER CENT
Contaminated on penicillin blood agar	17	2.4
Contaminated on blood agar	66	9.3
Contaminated on Lowenstein-Jensen	5	0.7
Total number of contaminated cultures	88	4.1

TABLE IV. COMPARISON OF RATE OF GROWTH

Average time of first appearance on penicillin blood agar	18.2 days
Average time of first appearance on blood agar	18.5 days
Average time of first appearance on Lowenstein-Jensen	18.7 days

TABLE V. COMPARISON OF RESULTS IN LITERATURE

	CONTAMINATION (PER CENT)			GROWTH INCIDENCE (PER CENT)				AVERAGE TIME OF FIRST APPEARANCE (DAYS)			
MEDIA	PBA	BA	L-J	PBA	BA	L-J	P	PBA	BA	L-J	P
Hosty et al.[9]	5.9	50.3	2.4	88.5*	88.2	94.3					
Preliminary series											
Final series	11.1		9.0	92.4		90.9					
Tarshis et al[5]	5.0		6.9	93.6		89.9		18.9		21.5	
Reinisch[6]					93.0*		90.0		23.4		26.6
Reichert[7]					85.6*	73.2			28.0	26.0	

PBA — Penicillin blood agar.
BA — Bloood agar.
L-J — Lowenstein-Jensen medium.
P — Petragnani.
*Excluding contamination.

DISCUSSION

While the rate of growth of *M. tuberculosis* was practically the same on all three media, the growth incidence on penicillin blood agar compared favorably with that on Lowenstein-Jensen and plain blood agar medium. The addition

of penicillin reduced the rate of contamination considerably, although contamination was still slightly higher on penicillin blood agar than it was on Lowenstein-Jensen medium.

In these experiments it was observed that blood agar showed a greater tolerance for variations in the pH of the inocula than was the case with Lowenstein-Jensen medium. This was indicated by the fact that inocula to which too much acid had been added, in the process of neutralization, yielded more positive cultures on blood agar than on Lowenstein-Jensen medium.

In contrast to our findings, there are reports which indicate a somewhat higher rate of contamination on penicillin blood agar medium. However, in these experiments the material used for culture was from 3 to 7 days old, while ours were all specimens not older than 24 hours.[5, 9]

Results similar to those presented here have been reported elsewhere.[5, 6, 7, 9] The reason or reasons for the satisfactory growth on blood media have been laid to the presence of hemoglobin[7] in the medium. Thus, Frisch[10] found hemoglobin to be one of the most important nutrients capable of supporting the growth of *M. tuberculosis* from small inocula. Furthermore Fisher[11] demonstrated that hemin was necessary for the growth of some strains of *M. tuberculosis* which were made resistant to INH in vitro. We included 14 known INH resistant strains from patients in our own study but this small number did not show any consistency in preference for blood or egg yolk media.

Our experiments seem to show that penicillin, when used in blood agar media, does not inhibit growth of tubercle bacilli.

This again was in accord with findings by others who used a variety of other media.[12-15] However, in a few instances, when special methods were used, *M. tuberculosis* was inhibited by small amounts of penicillin, for instance in Tween albumin medium.[14] This was believed to be due to the action of the Tween albumin causing greater contact of the antibiotic with the bacilli. Moreover, penicillin also seemed inhibitory to small inocula of some strains in a slide culture method.[16] It was thought that in these experiments inhibition was due to the fact that its level was maintained and that the experiment was terminated after 7 days. In other media there is a gradual falling off of the effect of penicillin, and growth is usually not obtained until two or more weeks have elapsed following inoculation. This, therefore, provides for inhibition of contaminants in the beginning and does not seem to effect the later growth of *M. tuberculosis*. Solotorovsky[17] also reported inhibition of very small inocula of tubercle bacilli, when using the liquid Dubos medium. Furthermore, some inhibitory effects of penicillin on *M. tuberculosis* were also found[18-20] when penicillin was used in addition to some other inhibitory agents, such as malachite green, and in these experiments the effect was probably synergistic.

Since it has been shown repeatedly that some strains of *M. tuberculosis* show selectivity for certain media, it appears advisable that other standard media be used in addition to penicillin blood agar medium. The preparation of penicillin blood agar is simple and low in cost. Therefore, this medium represents a valuable addition to the group of media now used for the cultivation of the tubercle bacillus.

SUMMARY

Penicillin blood agar was used for routine diagnostic cultures of *M. tuberculosis*. The incidence of growth was slightly higher than it was on Lowenstein-Jensen medium, while the time of first appearance of growth was the same. Contamination on penicillin blood agar was considerably lower than on blood agar medium without penicillin.

Penicillin blood agar is low in cost and easily prepared. It represents a valuable addition to the group of media used for the culturing of *M. tuberculosis*.

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